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## ERRATA

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Page 411, second line from bottom. for 1.5 per cent read 0.15 per cent.

“ 419, top line, for 0.1, read 0.01 to, etc.

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Resumen por el autor, Tadachika Minoura,  
University of Chicago.

Un estudio de los injertos de testículo y ovario en el huevo de gallina, y sus efectos sobre los embriones.

Injertando un trozo de testículo u ovario de gallina en la membrana corio-alantoidea de embriones en vías de desarrollo, el autor ha obtenido un cierto número de casos en los cuales creció el injerto (con anastomosis de los vasos sanguíneos embrionarios), y los embriones presentaron caracteres intersexuales en el sistema reproductor. Antes de salir del huevo, un macho normal presenta testículos de igual tamaño y un conducto de Wolff a cada lado; la hembra normal posee un solo ovario (izquierdo), dos conductos de Wolff y dos canales de Müller, el izquierdo presentando ya una diferenciación en ostium en el extremo anterior y otra en glándula productora del cascarón en el extremo posterior.

Los caracteres intersexuales que aparecen en varios grados en los embriones afectados por injertos de gonadas son: (1) Coexistencia de gonadas de tipo masculino con conductos de Müller diferenciados, de tipo hembra. (2) Gonada más grande en el lado izquierdo y gonada más pequeña en el derecho; estas gonadas son de tipo macho, pero en lo referente a su tamaño presentan un rasgo propio de la hembra. (3) Persistencia de la gonada derecha en embriones de tipo hembra. Los embriones normales en los cuales se han injertado otros órganos (hígado, bazo, tiroides y timo) no presentan los caracteres descritos. Estos experimentos demuestran que materiales específicos (hormones sexuales) segregados por el testículo y ovario estimulan el desarrollo y la diferenciación de los órganos sexuales homólogos e inhiben los del sexo opuesto. De este modo se ha podido influir en cierto grado sobre la diferenciación sexual y la reversibilidad de los caracteres sexuales primarios de un modo experimental, en el embrión de pollo.



A STUDY OF TESTIS AND OVARY GRAFTS ON THE  
HEN'S EGG AND THEIR EFFECTS ON THE  
EMBRYO

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ONE TEXT FIGURE AND TEN PLATES (THIRTY-SIX FIGURES)

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## INTRODUCTION

The study of the free-martin by F. R. Lillie ('17 b) has thrown new light on the field of the biology of sex, demonstrating that sexual differentiation is controllable, at least to some extent, by a certain physiological factor. Lillie discovered that in the free-martin, the female of two-sexed twins in cattle, the sex-glands and sex-ducts are modified from the normal female condition to a sterile masculinized state; that in cattle twins an anastomosis of the umbilical blood-vessels exists between the two embryos, and that this anastomosis is responsible for the alteration of the female twin, the sex-hormones from the male twin being thus enabled to gain access to the female twin through the blood current and to produce a profound modification of her reproductive system. A histological study of the same material by Chapin ('17) supported Lillie's conclusion that the free-martin is a modified female. As Lillie suggested ('17 a), "the possibility exists, however, that definitely planned experiments may enable us to regulate time and dosage of hormones better than is done in this experiment of nature; the results of such experiments cannot of course be foreseen. . . . Such experiments will be necessary for the full solution of the stated problem." I therefore, at Professor Lillie's suggestion, undertook to perform such a series of experiments for the purpose of determining whether or not sexual differentiation is experimentally controllable through the secretion of the sex-glands. Gonads were grafted on the membranes of developing chick embryos in relatively young stages; such embryos were then allowed to develop further, and in cases where the grafts grew, the urinogenital systems of the embryos were studied to determine what effects had been produced by the presence of the gonad grafts. The results of these experiments are presented in the present paper and others to appear subsequently.

It has already been abundantly proved through numerous studies on birds and mammals that the sex-glands do produce a secretion or secretions which play an important rôle in the production and maintenance of secondary sexual characters. This literature is so familiar to every one that it will not be reviewed

here. With reference to birds, it may merely be said that Goodale ('16) and Pezard ('18) have demonstrated in the most convincing manner that certain secondary sexual characters of domestic birds are dependent on the secretions of the gonads.

Varying degrees of reversal of certain sex characters have been observed or experimentally obtained in both vertebrates and invertebrates by several investigators. Thus Steinach ('12, '13) described a reversal of several sex characters in the guinea-pig and rat produced by transplanting the gonads of the opposite sex into castrated or spayed individuals. His statements have been partially verified by Moore ('19). These experiments show that in these mammals certain sexual characters, both somatic and psychic, are controlled by the internal secretions of the gonads. Riddle ('16) found that in certain crosses between pigeons intersexual forms arose in correlation with a certain phase of the breeding cycle. Pearl and Boring ('18) investigated several hermaphroditic fowls and found that they possessed pathological gonads, which were probably responsible for the hermaphroditic condition. Bond ('14) reported an interesting case of a hermaphroditic pheasant of the Formosan variety which displayed male secondary characters on the left side of its body and female characters on the right side. The bird possessed a single gonad, the left one, and this was in part testicular, in part ovarian. Bond believed hormones secreted by the hermaphroditic gonad were responsible for the external hermaphroditism.

Among invertebrates the studies of Baltzer, Goldschmidt, and Gould are of great interest. Baltzer ('14) found that in the Gephyrean worm *Bonellia*, the indifferent larva becomes a male only when it attaches itself to a female; otherwise it develops into a female. A closely similar case was described by Gould ('17, '19) for *Crepidula*. In this protandric gasteropod, maleness develops only when small individuals come in contact with or remain within a short distance of a large female or transitional form. Gould was convinced by his experiments that the development of male characters is directly due to the effect on sexually indifferent individuals of a certain substance produced by large animals. Baltzer and Gould did not claim that the substances



produced by the females in these cases correspond to the sex-hormones of vertebrates, but their function is evidently much like that of hormones. Goldschmidt ('16) has described intersexual forms in hybrid gypsy-moths and attempted to account for them by postulating a quantitative gradation in the value of the sex-determining factors. In his materials there was further a correlation between the degree of development of the secondary sexual characters and the degree of development of the gonads.

From investigations of this kind, two general conclusions may be drawn: first, that the sex-glands secrete certain substances which may conveniently be designated sex-hormones, which are related in an important way to the sexual characters; secondly, sexual differentiation is controllable, at least to some extent, by non-genetic factors.

The following experiments were carried out at the University of Chicago during the years 1917 to 1919. I am deeply indebted to Professor Lillie for his invaluable suggestions and criticisms during the course of the work and for his generosity in supplying me with numerous materials used in the experiments. I also wish to express my thanks to Dr. L. H. Hyman for revising the manuscript.

## GRAFTS

### *1. Material*

The common fowl was the material selected for the experiments, owing to the ease with which it can be operated on in embryonic stages. The white Wyandotte breed was employed in the majority of the experiments. The chicks from which the organs to be grafted were taken also belonged to this breed with a few exceptions in which other varieties of fowls were used.

### *2. Experimental method<sup>1</sup>*

*a. Operation.* The surface of the developing egg selected for operation was cleaned with dilute sublimate solution or 70 per

<sup>1</sup> This method was originally devised by Peebles ('08). It was later modified in several particulars by Murphy, Rous ('11), and Kiyono ('17).

cent alcohol with or without iodine. A small piece of shell, generally 10 to 15 mm. square was then cut out from the sterilized surface, leaving the egg membranes intact. Then a V- or U-shaped slit was made through the shell membranes by means of the very sharp point of an insect pin, sterilized in the flame. The cut edge of the membranes was lifted up and a piece or sometimes pieces of the organ to be grafted placed on a desirable spot on the surface of the chorio-allantoic membrane by means of the pin point. It is probable that in this operation the chorio-allantoic membrane was injured to some extent in the majority of cases. It was observed that the most favorable place for inserting the grafts is at the junction of blood-vessels on the chorio-allantoic membrane. As quickly as possible after placing the graft, the slit in the egg membranes was closed, and the piece of shell which had been removed replaced and sealed with paraffin. Sometimes a piece of paper previously immersed in melted paraffin was used to close the opening with equally good results. The operated egg was then returned to the incubator for further development.

Previous to such an operation it was of course necessary to prepare the organ which was to be grafted. Organs were removed from a chick and cut into small pieces about 1 mm. square. These pieces were kept moist in a Petri dish until the egg had been prepared for receiving them.

All of the instruments employed in the operation were sterilized in the autoclave.

The grafted organs were generally gonads. However, other organs, such as thymus, thyroid, spleen, liver, kidney, and others, were also grafted upon developing eggs in order to compare their effects upon the embryos with the effects produced by the gonads.

In each experiment generally ten to thirty eggs were operated upon and grafts from a gonad placed upon their chorio-allantoic membranes. A few control eggs were incubated simultaneously with each such lot. Some of these control eggs were treated in the same way as the operated eggs, a piece of paper or paraffin or shell being placed upon the membrane instead of a piece of organ; in other cases a window was made and closed without



inserting anything on the membrane; and in still other cases the control eggs were untreated.

*b. Examination.* The operated eggs were removed from the incubator at different intervals after operation for examination. Such intervals varied from one day to time of hatching. In spite of the greatest care, in some experiments many eggs became infected and failed to develop further. Observations and measurements were made upon the grafts and embryos in the living state; they were then preserved for further examination. Formol-Zenker was usually employed as a fixing agent, Bouin's fluid in some cases. Many of the preserved embryos were photographed after dissection. I am greatly indebted to Doctor Bartelmez for his generosity in permitting me to use the dark room in the Anatomy Department for photographing the materials, and to Dr. Marion Hines and Mr. K. Toda for their kind assistance and advice in making the photographs.

### 3. Growth of grafts

*a. External observations.* When an operated egg is opened for examination, if the grafted piece is growing or alive, a white mass will be found on the chorio-allantoic membrane, to which umbilical blood-vessels are connected (figs. 1, 4). The mass is somewhat translucent and white or yellowish or more or less rosy in color. The grafts in general grew much more readily toward the inside than the outside of the membrane (figs. A, 5). In several instances a marked convergence of blood-vessels to the graft was noticed. Vascular connection is established with the grafts as early as twenty-four hours after operation.

The amount of growth which has occurred in the grafts is of course different in different cases depending upon the following factors: 1) duration of the graft; 2) age of the embryo at the time of the operation; 3) degree of vascular connection with the grafts. Under the most favorable conditions, the grafted masses attained a considerable size, 10 mm. in diameter and several millimeters in thickness. In the majority of cases, however, they varied from 3 to 7 mm. in diameter, that is, the original

grafted piece had increased three to ten times in diameter. In table 1 are recorded a number of measurements of the size attained by grafted pieces of ovary, testis, and other organs.

TABLE 1

*Length and width of grafts of ovary, testis, and other organs implanted on the chorio-allantoic membrane of the chick embryos*

OVARY		TESTIS		OTHER ORGANS		
Number of embryo	Size	Number of embryo	Size	Number of embryo	Organ	Size
	<i>mm.</i>		<i>mm.</i>			<i>mm.</i>
33-6	12.0 x 5.0	43-1	9.0 x 4.5	29-1	Liver	14.0 x 7.0
30-9	9.0 x 7.0	43-3	9.0 x 4.5	30-7	Thyroid	6.5 x 5.0
44-15	7.0 x 4.0	22-14	9.0 x 4.0	27-1	Thymus	6.0 x 4.5
29-11	7.0 x 3.5	22-13	8.0 x 7.0	29-8	Kidney	5.5
44-4	6.5 x 3.5	30-2	8.0 x 2.0	29-9	Thyroid and	5.0 x 2.5
27-4	6.0 x 5.0	22-6	7.5 x 5.0		thymus	
19-2	6.0 x 5.0	22-12	7.0 x 5.0	29-4	Thymus	3.0 x 2.0
18-9	6.0 x 3.0	45-21	7.0			
19-1	5.0 x 5.0	22-9	6.0 x 5.0			
44-11	5.0 x 4.0	22-11	6.0 x 4.0			
17-1	5.0 x 3.0	8-5	5.0 x 4.0			
44-16	5.0 x 3.0	12-2	5.0 x 3.0			
44-14	4.5 x 2.5	47-4	5.0 x 2.5			
1-1	4.0 x 4.0	15-5	4.5 x 2.5			
44-3	4.0 x 3.5	45-6	4.5 x 2.0			
44-1	4.0 x 3.0	20-1	4.0 x 4.0			
7-1	4.0 x 3.0	22-14	4.0 x 4.0			
29-12	3.5 x 3.0	45-23	4.0 x 3.0			
41-7	3.5 x 2.5	31-3	4.0 x 2.5			
1-7	3.0 x 2.0	47-10	4.0 x 2.5			
6-2	3.0 x 2.0	30-8	4.0			
29-7	3.0 x 1.5	17-7	3.5 x 2.0			
29-5	3.0 x 1.5	34-5	3.0 x 3.0			
30-6	2.5 x 2.0	27-5	2.0 x 2.0			
42-6	2.5 x 1.8	45-15	2.0 x 2.0			
41-9	2.5	28-8	2.0 x 1.5			
17-5	2.0 x 2.0	45-18	2.0 x 1.0			
7-4	2.0 x 1.0	27-6	2.0 x 1.0			
2-5	1.5 x 1.0	30-4	1.5 x 1.5			

In table 2 the sizes attained by ovary and testis grafts are compared. From these tables it is evident that testis grafts grow somewhat larger than ovary grafts and that grafts of other organs undergo a greater growth than grafts from either gonad.



Kiyono ('17) grafted tissues and organs on chick and duck embryos and classified them according to their capacities for growth into three groups, the first named having the greatest capacity: 1) connective tissue, cartilage, skin; 2) muscle, mucous membrane of the alimentary and respiratory tracts; 3) kidney, eye, liver, nervous tissue, and glands. He stated that tissues and organs having simple functions grow most readily when grafted, while those having more complex structure and functions grow less easily. This statement was verified in my experiments, since I found that ovary and testis grafts have a lower growth capacity than thymus, liver, and spleen grafts.

TABLE 2  
*Comparison of sizes attained by testis and ovary grafts*

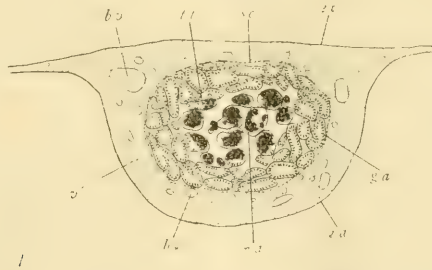
SIZE	NUMBER OF TESTIS GRAFTS	NUMBER OF OVARY GRAFTS
<i>mm.</i>		
Over 10	0	1
9-10	3	1
8-9	2	0
7-8	3	2
6-7	2	4
5-6	3	4
4-5	9	5
3-4	2	6
2-3	5	5
1-2	1	1

*b. Histological study.* The histological structure of sectioned grafts was studied with great interest. In general, sections of the grafts exhibited four distinct regions arranged concentrically (fig. A).

1. Chorio-allantoic membrane. The graft is encircled by the chorio-allantoic membrane. The external layer of this membrane consists of the ectoderm of the chorion; it passes over the outer surface of the graft (fig. A, *ec*). The internal layer passing beneath the graft is the entoderm of the allantois (fig. A, *ea*). Both ectodermal and entodermal membranes are very thin layers, one cell in thickness. Between the ectoderm and entoderm is the mesoderm, composed of the fused mesoderms of both chorion

and allantois. The graft lies in this mesoderm. The chorio-allantoic membrane appears in general nearly normal in structure, but is irregularly bounded and its cells somewhat more compact than is normally the case.

2. Vascular area. Within the membranes just described and encircling the graft proper is a vascular layer. This consists originally of a stroma of mesoderm cells richly infiltrated with small blood-vessels, but with the growth of the graft the stroma is greatly increased in quantity. It is composed of spindle-shaped connective-tissue cells and of fibers; it is somewhat more loose in structure than the normal stroma. Blood-vessels are



Text fig. A Diagram of the structure of a growing graft (testis). *ec*, ectoderm of chorion; *ea*, entoderm of allantois; *bv*, blood-vessel; *va*, vascular area; *ga*, growing area; *sc*, connective-tissue sheath; *na*, necrotic area; *tt*, transitional tubules, regenerating at one end and necrotic at the other.

distributed abundantly through the stroma and show a tendency to aggregate toward the center of the graft. Intermixed with the stroma structure one finds numerous leucocytes, singly or in groups. Some of these are hemoblasts or lymphoblasts; others are granuloblasts, granulocytes, or polymorphonuclear leucocytes. Many of them contain granules which stain with hemotoxylin and eosin; these are polymorphonuclear. Mitotic figures are not uncommon. Of course this abnormal accumulation of leucocytes around the graft is a response to the presence of foreign tissue.

3. Growing area of the graft. This is naturally the most important region of the graft—the area occupied by the growing tissues of the graft. In the case of testis graft one finds in this



area numerous growing seminiferous tubules, having normal cell arrangement and appearance. Mitotic divisions in the cells of the seminiferous tubules are readily and frequently observable in these testis grafts and their presence demonstrates beyond doubt that an active growth of the seminiferous tubules had occurred (fig. 6). Proliferation of endothelial cells of the stroma between the tubules is also often observable. In some cases around this new growth, that is, between it and the surrounding mesenchymatous stroma, a sheath of connective tissue, resembling the normal albuginea, had been formed (fig. A, *sc*). Study of serial sections showed that the seminiferous tubules had already assumed the convoluted form.

In the case of ovary grafts, the growing area consists of numerous follicles, each composed of a central ovocyte surrounded by a single layer of cuboidal granulosa cells, very much as in the normal ovary (figs. 2, 3).

4. Necrotic area. This consists mainly of a mass of necrotic tissue, but there is no distinct boundary between this and the preceding area. The size of the necrotic center varies in different grafts. As the new growth is always due to a regeneration from the original grafted piece of tissue, one finds, as a rule a gradual transition from the center of the necrotic area to its periphery, from necrotic to living tissue (fig. A, *tt*). Thus in testis grafts one end of a tubule will consist of living or regenerating cells, while the other end terminates in a degenerating mass. The necrotic changes involved in many cases pycnosis or karyorrhexis, in other cases, karyolysis. The degree of necrosis developed in the center of the graft is dependent of course upon the rapidity with which a vascular supply to the graft develops. The slower the establishment of vascular and nutritive connections between the graft and the embryo, the more extensive are the necrotic changes in the graft. In some cases the central tissue mass was almost liquified and absorbed by phagocytosis, as was demonstrated by the existence of giant-cells around the tissue debris. The position and number of necrotic centers varies in different grafts, and on this basis the grafts may be classified as: monocentric, in which case the necrotic area may be either centrally located or acentric, dicentric, and polycentric.

*c. Comparison of growth of testis grafts and ovary grafts.* It was generally found that testis tissue grew faster and more readily than ovary tissue. Although the ovary grafts as a whole exhibited considerable growth, the amount of new growth, that is of follicular ovarian tissue, was rather small as a rule. In the case of testis grafts, on the contrary, a well-marked new growth of testis tissue was often found, in spite of the smaller size of the grafts as a whole. In other words, ovary tissue shows a greater tendency toward necrosis and less tendency toward growth and differentiation than testis tissue. We cannot at

TABLE 3

*Showing age of the embryos at the time of grafting and number of embryos of each age which were affected by the presence of the graft*

AGE OF EMBRYOS AT TIME OF OPERATION	NUMBER OF EXPERIMENT	NUMBER OF AFFECTED EMBRYOS		
		Markedly affected embryos	Slightly affected embryos	Total
<i>days</i>				
2	35	0	0	0
4	9, 25, 29, 36	0	1	1
5	8, 35	1	2	3
6	28, 42, 43	1	1	2
7	2, 3, 7, 26, 32, 45, 46	7	7	14
8	1, 18, 20, 21, 36, 41, 47	3	17	20
9	10, 27, 30, 31, 35, 40	7	3	10
10	15, 33, 44	2	12	14
11	14, 19, 22, 28, 29, 34	5	14	19
12	46	0	0	0
13	17, 18	4	0	4
16	30	0	0	0

present give any explanation for this difference. It may be suggested, however, that under the same physiological conditions ovary tissue requires a more rapid or more abundant nutritive supply than testis tissue, or that ovary tissue is less resistant to a foreign environment than testis tissue, or that both factors are are responsible.

*d. Age of the embryo most suitable for grafting.* The organs which were grafted were taken from birds of various ages, from nine-day-old embryos up to adult fowls. The ages of the embryos upon which the grafts were implanted are given in table 3.



As shown in table 3 the grafts produced the most effect when they were implanted on the embryos during the second week of incubation, that is, from the seventh to the thirteenth days of incubation. Especially 8-, 9-, 10-, and 11-day old embryos were found to be most favorable for the purpose of the experiments. The facts revealed by table 3 are not difficult to understand. In embryos younger than five days the allantoic circulation is not sufficiently well established to permit of vascular connections with the graft; grafts implanted on such young embryos therefore die from a lack of blood supply. In the case of embryos more than two weeks old it is probable that a resistance to foreign tissues has been developed and that such embryos are thereby enabled to destroy the grafts. Murphy ('14) pointed out that a defensive mechanism against foreign tissue develops in chick embryos at about the time of hatching. He noted a sudden appearance of lymphoid cells around the graft at this time and ascribed the function of the destruction of foreign tissue to these cells, considering them to be similar in function to the cells of the spleen and of bone-marrow. My results are in general agreement with Murphy's statements except that in the case of my experiments I am unable to determine whether the resistance to foreign tissue appears suddenly or develops gradually. It is, however, certain that embryos more than two weeks old are unfavorable for the implantation of foreign tissue.

#### ANATOMICAL STUDY OF THE EMBRYOS

We may now proceed to a consideration of the effects of the gonad grafts on the anatomy of the chick embryos. In this paper only the gross anatomy of the affected embryos will be described, the histological findings being reserved for a subsequent paper.

##### *1. Development of sex-glands and sex-ducts in normal embryos*

Before passing to a consideration of the experimental results, it is necessary to review the normal development of the sex-glands and sex-ducts in the chick, with special reference to those points concerned in the present experiments.

*a. Sex differentiation.* R. Semon ('87) described the differentiation of sex in the chick in the following words:

Die geschlechtliche Differenzierung, die sich beim Hühnchen am 5., spätestens am 6. Tage erkennen lässt, macht sich zunächst dadurch bemerklich, dass bei weiblichen Embryonen die rechte Keimdrüse in auffallender Weise im Wachstum zurückbleibt. Auch beim Männchen entwickelt sich häufig der rechte Hode langsamer und bleibt oft zeitlebens kleiner. Aber die Differenz ist hier stets so unbedeutend, dass man am 6. Tage immer ohne Mühe Hode und Eierstock an diesem Merkmal unterscheiden kann.

Later Swift ('15), on histological grounds, stated:

When the chick embryo has reached the 156th hour of development ( $6\frac{1}{2}$  days), the formation of cords of first proliferation ceases rather abruptly and it is about this time that the sex of the individual can be definitely determined. In the determination of sex there are three criteria on which reliance can be placed. These are the relative size of the two gonads, the germinal epithelium, and the number of primordial germ-cells in the germinal epithelium. . . . an interesting fact and one to which attention has not been previously called, is the presence of more primordial germ-cells in the germinal epithelium of the left female gonad than in the epithelium of the male gonad.

From these two quotations it is evident that the first signs of sexual differentiation, both micro- and macroscopic, appear between the fifth and seventh days of incubation. The most readily recognizable character is the difference in size between the right and left gonads in the female. Besides this size difference, a difference in shape between male and female gonads is also noticeable. In some cases the retrogression of the müllerian ducts in the male had already begun on the sixth day. We may therefore say in general that sexual differentiation appears toward the end of the first week of incubation.

*b. Degeneration of the right ovary.* Both ovaries develop equally until the end of the first week of incubation, that is to say, the development of the ovaries is symmetrical up to the time when sexual differentiation appears. But from this time on the development of the two ovaries is asymmetrical. The left ovary continues to increase in length and size, while the right ovary undergoes a process of degeneration and finally



disappears, usually before hatching. Although the degeneration of the right ovary in birds has been known for a long time, there are, curiously enough, no detailed studies upon the matter, so that there is in the literature no information available concerning the degree of development attained by the right ovary, the time at which it begins to retrogress, or the manner and degree of its retrogression. From my work I am able to add some details to the facts already known. After the stage when the difference between the two ovaries becomes apparent, the right ovary shows some slight increase in length, usually not more than 50 per cent. This continues until about the end of the second week of incubation. From this time on, a process of degeneration is manifested in the right ovary by its decrease in length and change in position. Up to the sixth or seventh day the two ovaries lie nearly parallel to the longitudinal axis of the body. After the right ovary has begun to degenerate, its anterior end tends to incline more and more to the right side, so as to assume an oblique position with reference to the right wolffian body. The outlines of the ovary gradually become less and less distinct; this change is more marked on the medial and anterior margins of the ovary. Meantime the volume of the ovary continually diminishes so that it becomes more and more slender. In the case of several 18 to 20-day-old females, the right ovary was reduced to a flattened membranous body, although the fading outlines could still be determined. The final disappearance of the right ovary occurs in general at about the age of 18 days; individual differences, of course, exist. The data on the length of the two ovaries at various stages of development are given in table 4.

*c. Wolffian ducts.* It has generally been believed that in the female chick embryo the wolffian ducts degenerate simultaneously with the wolffian bodies before hatching, or, at least, at some later time. The recent observations of Goodale ('16) Boring and Pearl ('18), however, show that this belief is erroneous. Goodale states that "apparently the wolffian duct and body may not always degenerate in the female." Boring and Pearl dissected a number of newly hatched chicks or chicks

TABLE 4  
*Length of right and left ovaries at various ages*

AGE OF EMBRYO	LENGTH OF LEFT OVARY	LENGTH OF RIGHT OVARY
<i>days</i>	<i>mm.</i>	<i>mm</i>
7	1.5	1.5
9	2.2	1.6
10	3.2	2.2
11	3.7	2.0
12	3.5	2.0
13	3.0	1.3
	3.0	1.5
	3.3	1.7
14	5.0	2.0
15	4.3	1.8
16	4.3	1.5
17	4.3	1.5
	4.3	1.5
	4.5	1.3
	4.6	1.5
	5.0	1.8
18	4.0	1.5
	4.8	0.0
	5.0	2.0
	5.0	2.0
	5.0	0.0
	5.0	0.0
	5.2	1.5
20	5.0	1.0
	5.2	0.0
	6.0	0.0



from pipped eggs, seven of which were female, and five laying hens, and found that all of them had wolffian ducts. My own observations agree with those of Boring and Pearl. I have examined hundreds of embryos, and in all females without exception, the wolffian ducts were present. The wolffian bodies are always found in a degenerating condition as the time of hatching approaches and in some cases have almost completely disappeared at this time, but the wolffian ducts persist.

It is therefore certain that the presence of the wolffian ducts cannot be used as a criterion of the male condition. Whether the wolffian ducts eventually disappear in very late adult life and whether they have some function during their existence in the female are not known at the present time.

A careful study was made to determine whether or not the wolffian ducts are equally developed during embryonic life in the two sexes and whether or not the right and left ducts in each sex are of the same dimensions. It was found that in the male the posterior portion of the wolffian ducts is generally of greater diameter than in the female. This difference is noticeable in the second week of incubation in most, if not all, cases. It was further observed that in both male and female embryos after the second week the left duct, especially near its posterior end, or near its connection with the cloaca is distinctly larger than the right duct. Sometimes the left duct of the male is nearly as large as the ureter, while the right duct is always smaller than the ureter. In the female the left duct generally runs dorsal to the müllerian duct near its posterior end and is bound to the latter by connective tissue.

These points of difference between the wolffian ducts of male and female, although slight, can nevertheless be utilized as distinguishing sexual characteristics.

*d. Müllerian ducts.* The fate of the müllerian ducts in embryonic life is quite characteristic in each sex. In the male these ducts on both sides do not develop very far, but gradually regress and always disappear before hatching. In his book "The Development of the Chick," Lillie states that "Retrogression begins posteriorly and proceeds in the direction of the head; the

ostium is the last to disappear." My observations agree in general with this statement. The process of retrogression does not proceed regularly from the posterior end, but begins in one or several places in the ducts irregularly so that intact portions are left between thread-like degenerated portions. The anterior end at the level of the wolffian body remains intact longer than other regions of the ducts in most embryos. The time at which retrogression begins is more or less variable; sometimes the process has already begun in six-day old embryos while in other cases one duct, but generally not both, may still persist in twelve or thirteen-day-old embryos in a nearly intact condition. In the majority of cases the right müllerian duct retrogresses more rapidly than the left one. Both ducts completely disappear between the seventeenth day of incubation and the time of hatching.

In the female, the left müllerian duct persists and continues to develop, eventually differentiating into a functional oviduct. The process of degeneration of the right duct is, however, quite different from that in the male.

The degeneration of the right müllerian duct was investigated by Gasser ('74). Gasser states that the duct ceases to develop soon after the eighth day; that by the twelfth day it is no longer present in the region of the sex-glands; by the fifteenth day it has disappeared from the region occupied by the wolffian body, and that after this time there remain only traces of the duct along its course together with a small lumen close to the cloaca. According to my observations, however, the degeneration of the duct does not take place as regularly as described by Gasser. In the first place it is somewhat doubtful that the development of the duct ceases after the eighth day; a differentiation of the posterior end, a widening similar to that occurring in the left duct, is always noticeable subsequent to this time. In fact, this differentiation of the posterior end may continue for a considerable length of time, even though degeneration is progressing at the anterior end. Consequently, in advanced stages, after retrogression is completed, the posterior end of the right müllerian duct is still present as a short widened tube with a small

lumen near the cloaca. This differentiation of the posterior end of the right müllerian duct of the female does not occur in the male, even to the slightest degree. In the second place, the degeneration was found to occur somewhat irregularly. In many cases, in embryos as young as eleven or twelve days, the right duct was already in an advanced state of degeneration. On the other hand, seventeen-day-old embryos may retain more than half of the duct intact.

*e. Range of size variation in normal testes.* Data on the normal size variation of the reproductive glands and ducts in birds are rather scanty. Almost no attention has been paid to the variation in the size of the two testes, it having apparently been assumed that the testes of the two sides develop equally.

Etzold ('91) measured the testes of the sparrow and found that the left testis is larger than the right one. Swift ('16) made the same observation in the chick embryo at the age of six days and noticed further that there are more germ cells in the left testis. Riddle ('17) studied this matter very carefully in the pigeon and found that the right testis normally weighs more than the left testis. In regard to the shape of the two testes, he states that "the left is thinner and more elongated, the right shorter and thicker." In regard to this difference in shape between the two testes, Riddle made an interesting suggestion: "This difference in form is perhaps not without interest since the only persistent gonad in the female—that of the left side—is characteristically 'thin' and 'long.' The testis that develops on this side is similarly characterized as compared with its mate of the right side." Riddle also determined the weight of the testes in the fowl, but owing to the small number of individuals investigated, the results were indefinite.

I have collected some data on the development, size, and shape of the two testes in chick embryos. Both testes develop rapidly until about the eighteenth day, after which their rate of development diminishes. They are about equal in size, or the left one may be a little larger. This inequality in the size of the two testes appears more clearly in the earlier stages of development and tends to disappear later. In the majority of cases, the



difference in length of the two testes ranges between 0.1 and 0.3 mm. In normal embryos the difference does not exceed 0.5 mm. The shape of the two testes is similar to that described by Riddle for the pigeon. The left testis is more elongated than the right one; the right one is shorter and in a few cases thicker. But in general I cannot say that the right testis is the larger of the two. Owing to the nature of my experiments, I could not remove the testes for the purpose of weighing them, but I am inclined to think that in the chick the left testis is larger and weighs more than the right one. The data concerning the lengths of the right and left testes of the chick are given in table 5.

## *2. Development of sex-glands and sex-ducts in grafted embryos*

*a. General résumé of the experimental results.* Nearly one thousand eggs were used in the experiments. Of these about three hundred were incubated as controls and about one hundred more were used for grafting organs other than gonads. The remaining eggs served for the implantation of pieces of ovary and testis. In all 187 embryos on which gonad grafts had been implanted were living at the time of examination, and these furnish the materials for this part of the paper.

The 187 embryos obtained in the experiments are classified in table 6. In this table and elsewhere testis graft means that a piece of testis had been grafted on the membranes of the embryo in question and ovary graft that a piece of ovary had been so grafted. Male-type embryos are those which have predominantly male anatomical characteristics. The normal male at or near the time of hatching is distinguished by the following characters: there are two testes of equal size; the testes are elongated in form and smooth in texture, and there are no müllerian ducts (figs. 7, 8). Affected embryos whose gonads and ducts approach this description are hence regarded as male-type embryos. Female-type embryos are those whose gonads and ducts resemble those of the normal female. The characteristics of the normal female at or near the time of hatching are: the right ovary is absent; the left one is larger and broader than either testis; its surface is commonly rough in texture; the left

TABLE 5

*Length of right and left testes of the normal chick embryo at different ages*

AGE OF EMBRYO	LENGTH OF LEFT TESTIS	LENGTH OF RIGHT TESTIS	DIFFERENCE IN FAVOR OF LEFT TESTIS
<i>days</i>	<i>mm.</i>	<i>mm.</i>	<i>mm.</i>
7	2.8	2.7	0.1
13	3.0	2.8	0.2
	3.0	3.0	0.0
14	3.0	3.1	-0.1
15	3.2	3.0	0.2
	3.2	3.3	-0.1
	3.7	3.5	0.2
16	3.2	3.3	-0.1
17	3.0	3.0	0.0
	3.0	3.0	0.0
	3.7	3.5	0.2
18	3.3	3.3	0.0
	3.7	3.8	-0.1
	3.8	3.5	0.3
	3.8	4.0	-0.2
	4.0	4.0	0.0
	4.7	4.5	0.2
19	4.3	4.0	0.3
20	4.0	4.0	0.0
	5.0	5.0	0.0
21	4.0	4.0	0.0

TABLE 6

*Classification of all experimentally obtained embryos*

	MARKEDLY AFFECTED EM- BRYOS (INTER- SEXES)	SLIGHTLY AFFECTED EMBRYOS		APPARENTLY NON- AFFECTED EMBRYOS		TOTAL
		Male type	Female type	Male	Female	
Testis grafts . . . . .	16	13	20	23	32	104
Ovary grafts . . . . .	14	15	10	17	27	83
Total . . . . .	30	58		99		187

müllerian duct is greatly enlarged and differentiated in the direction of the adult oviduct; the terminal portion of the right müllerian duct is generally still present (fig. 9, 10). Affected embryos whose gonads and ducts are similar to this condition are regarded as female-type embryos. It is to be understood that the gonads and ducts of the affected embryos exhibited all possible gradations between apparently normal embryos and intersexual conditions, in which cases, the embryos could not be definitely assigned to either sex.

In table 6 the experimentally obtained embryos are classified into three categories: markedly affected embryos, slightly affected embryos, and those apparently non-affected. Since, however, all gradations exist between these three classes, it is to be understood that the assignment of some of the embryos into such categories is largely arbitrary.

In general it may be said that whenever the grafts exhibited a good growth, the embryos were affected, but when the grafts grew slightly or not at all the resulting embryos were nearly normal or apparently non-affected.

I shall now describe in detail the anatomical characters of a number of affected embryos, selecting those which showed distinct alteration of their urinogenital systems toward an intersexual condition. In these descriptions each embryo is designated by a double number, the first one referring to the number of the experiment, the second to the number of the particular embryo in that experiment. For instance, embryo 27-6 designates the sixth egg operated on in the twenty-seventh experiment.

*b. Description of embryos affected by testis grafts.*

1. Embryo no. 27-6 (fig. 11, 12). Operated May 7, 1918. One-fifth of the left testis of a one-month old chick was grafted on the membranes of an embryo nine days old. Testis 5 mm. long; grafted piece about 1 mm. square. Examined May 17th; embryo developing and graft with vascular connections, of small size, however. Age of embryo, 19 days; duration of graft, 10 days.

Result: Right gonad of affected embryo 4.1 x 1 mm.; left gonad 4.1 x 0.8 mm.; peculiar black pigmentation over half of



left gonad. Wolffian ducts of male type; müllerian ducts present on both sides. The left müllerian duct is about 20 mm. long; right about 9 mm., the anterior part of the right duct having disappeared as in the female. The posterior portions of both ducts are distinctly expanded; the expanded portions of the left duct measures  $7 \times 1.5$  mm., that of the right duct,  $5 \times 1.2$  mm. The ostium of the left duct and the differentiation of its posterior part into uterus and shell gland are nearly the same as in the normal female. But at least two parts, the one near the posterior end of the left wolffian body and the other about 3 to 4 mm. anterior to the expanded portion, show slight signs of degeneration; that is to say, decrease in diameter of the duct. It is evident, then, that this embryo possesses müllerian ducts of the female type but gonads of the male type. It is therefore in a typical intersexual condition.

2. Embryo no. 31-3 (fig. 13, 14). Operated June 14, 1918. Both testes (left 3.8 mm. long, right 4 mm. long) of a two-weeks-old chick were grafted on the membranes of an embryo of eight and one-half days' age. Opened June 24th. The two grafts were found with vascular connections. Age of the embryo, nearly 19 days; duration of graft, 10 days.

Result: Gonads of the male type, both 3.5 mm. long. Müllerian ducts present on both sides, the left one 22 mm., the right one 20 mm., in length; posterior portions of both show signs of enlargement. The right duct gradually increases in size posteriorly and the left one shows marked posterior differentiation. A short middle portion of the left duct exhibits signs of degeneration. This embryo, like the preceding one, has gonads of the male type, but the general condition of the müllerian ducts is decidedly female-like, although not so strikingly so as in the preceding case.

Attention may be called to the fact that in these two embryos, nos. 27-6 and 31-3, which are the most highly modified ones obtained, the gonad graft was implanted on the embryo when the latter was in a relatively early stage of sex differentiation.

3. Embryo no. 47-10 (fig. 15). Operated July 19, 1919. Two pieces of testis from a week-old chick were grafted on an

eight-day embryo. Opened August 1st. Grafts growing, but small. Age of embryo, 21 days; duration of graft, 13 days.

Result: Gonads of the male type, the left one  $3.5 \times 1.1$  mm., the right one  $3 \times 1$  mm. They have, however, an abnormal position on the wolffian bodies; instead of being parallel to the median sagittal axis, they are inclined 50 to 60 degrees to it. Wolffian ducts small, of the female type. Both müllerian ducts present, and both with slight posterior differentiation as a uterus. This embryo has male-like gonads, but female type ducts.

4. Embryo no. 43-3 (fig. 16). Operated March 14, 1919. Piece of testis about 1 mm. square from a chick thirty-six days old implanted on an embryo of six days. Opened for examination March 29th. Graft showed good growth. Age of embryo, 21 days; duration of graft, 15 days.

Result: Gonads of the male type; left one 3.5 mm. long; right 5.7 mm.; anterior two-fifths of the left gonad pigmented. Wolffian ducts rather of the male type; müllerian ducts present on both sides and exhibiting only slight expansion of their posterior portions.

5. Embryo no. 27-5 (fig. 17). Conditions of the experiment same as under embryo no 27-6.

Result: Gonads of the male type; left,  $3.7 \times 1.4$  mm.; right,  $3 \times 1.3$  mm. Wolffian ducts of the male type. Anterior portions of the müllerian ducts present; posterior two-fifths of their courses degenerating on both sides. Such persistence of the anterior portions, and the shape of posterior portions (although they are retrogressing) show female rather than male characteristics, or at least indicate an original development in the female direction later inhibited.

6. Embryo no. 28-8. Operated May 18, 1918. The right testis of a chick embryo one week old was grafted on an embryo of eleven days. Opened May 27th. Age of embryo, 20 days; duration of graft, 9 days.

Result: Gonads of the male type; left  $4.5 \times 1.1$  mm.; right,  $3.5 \times 1.3$  mm. Wolffian ducts of the male type. The müllerian ducts have degenerated, leaving slender thread-like remnants along their original courses. As a whole this embryo is more

male-like than those described already; but the size relations of the two gonads is abnormal, the left one being larger than the right.

7. Embryo no. 45-18 (fig. 20). Operated May 1, 1919. Piece of adult testis (bird six or more months old, testis 18 mm. long) grafted on a seven-day-old embryo. Opened May 13th. Age of embryo, 19 days; duration of graft, 12 days.

Result: Gonads of the male type, but the left one is very much larger than the right: size of left gonad, 4.3 x 1.2 mm.; size of right one, 3 x 1.2 mm. The wolffian ducts are not very characteristically male. A slight trace of the anterior part of the left müllerian duct present; posterior part only of the right duct present and attached to the cloaca as in the normal female.

8. Embryo no. 45-6 (fig. 19). Experimental conditions the same as in no. 45-18.

Result: Gonads of the male type, but the left one larger than the right, as in no. 45-6; left gonad, 4.2 x 1 mm.; right gonad, 3 x 1 mm. Wolffian ducts more or less of the female type; only traces of the müllerian ducts present.

9. Embryo no. 15-5 (fig. 18). Operated November 13, 1917. Piece of adult testis grafted on an embryo ten days old. Opened November 22nd. Age of embryo, 19 days; duration of graft, 9 days.

Result: Left gonad of the male type, 5 x 0.9 mm.; right gonad more or less of the female type, 2.7 x 1 mm. Wolffian ducts of the male type. Posterior portions of the müllerian ducts present on both sides, about 5 mm. in length; the left duct somewhat differentiated in the female direction.

10. Embryo no. 10-5. Operated October 3, 1917. Piece of adult testis grafted on a nine-day-old embryo. Opened October 23rd. Age of embryo, 19 days; duration of graft, 10 days.

Result: Right gonad of the male type, 3 mm. long; left gonad not typically male in form, but flattened somewhat like the female type gonad, 4.8 mm. long. Left gonad markedly larger than the right. Wolffian ducts of the male type. Condition of the müllerian ducts could not be made out, owing to poor preservation.



11. Embryo no. 45-23. Conditions of the experiment the same as in nos. 45-18 and 45-6. The graft exhibited marked growth and possessed well-established vascular connections.

Result: Gonads of the male type; left gonad, 3.3 x 0.8 mm.; right gonad, 2.8 x 0.7 mm. The left is longer and the texture of its surface different from that of the right. Wolffian ducts rather of the male type; no trace of the müllerian ducts.

12. Embryo no. 8-5. Operated August 29, 1917. Adult testis grafted on an embryo of five days. Opened September 11th. Age of embryo, 18 days; duration of the graft, 13 days.

Result: Gonads of the male type; left gonad, 3.5 mm., right gonad 2.5 mm. in length. Wolffian ducts of the male type. Anterior portion of the right müllerian duct and posterior portion of the left one present.

13. Embryo no. 22-13 (fig. 22). Operated December 19, 1917. Piece of adult testis grafted on an embryo of eleven days. Opened December 26th. Graft showed good growth, 7 x 8 mm. in dimensions. Age of embryo, 18 days; duration of graft, 7 days.

Result: Gonads of the female type in general; left gonad, 4.5 x 1.5 mm.; right gonad, 3 x 1 mm. The right gonad is markedly larger than is the case in the normal female of this age. Wolffian ducts of the male type; müllerian ducts of the female type.

14. Embryo no. 17-7 (figs. 23, 24). Operated November 19, 1917. Two pieces of testis from a week-old chick were implanted on a thirteen-day-old embryo. Examined November 26th. Age of embryo, 19 days; duration of graft, 6 days.

Result: Gonads of the female type; left gonad, 4.5 x 1.7 mm.; right gonad, 3.3 x 0.5 mm. Right gonad larger and more distinctly outlined than in normal females of the same age. Wolffian ducts more or less of the female type; müllerian ducts of the female type, but their posterior ends not differentiated to the normal degree.

15. Embryo no. 45-15. Conditions of the experiment the same as in nos. 45-18 and 45-6.

Result: Gonads of the female type, but right gonad larger than is normally the case; left gonad, 4.5 x 1.8 mm.; right gonad, 2.3 x 0.8 mm. Wolffian ducts more or less of the male type; müllerian ducts of the female type.

16. Embryo no. 22-9. Conditions of the experiment the same as in no. 22-13. The graft grew well. Age of embryo, 19 days; duration of graft, 6 days.

Result: Gonads of the female type; left gonad 6 mm. long; right gonad, 3 mm. long. Wolffian ducts of the male type; müllerian ducts of the female type.

*c. Description of embryos affected by ovary grafts.*

1. Embryo no. 27-4 (figs. 25, 26). Operated May 7, 1918. Piece of ovary about 1 mm. square from a chick one month old was grafted on a nine-day-old embryo. Examined May 17th. Graft with vascular connections, but rather small. Age of embryo, 19 days; duration of graft, 10 days.

Result: Gonads of the male type, but ducts of the female type. Right gonad, 3.2 x 1.4 mm.; left gonad, 4 x 1.2 mm. Gonads testis-like in appearance, but the left one much the larger. Both müllerian ducts abnormal; the posterior portions were distinctly differentiated as in the female; there were no signs of degeneration in the ducts such as occur in normal males. This embryo is typically intersexual.

2. Embryo no. 7-3 (fig. 27). Operated August 25, 1917. Piece of adult ovary grafted on a seven-day embryo. Opened September 4th. Age of embryo, 17 days; duration of graft, 10 days.

Result: Right gonad of the male type, 2.8 x 0.6 mm.; left gonad of the female type, 3.6 x 1 mm. Left gonad considerably larger than the right; its surface not smooth as in the normal testis, but rough like an ovary. Ducts poorly preserved. Parts of the müllerian ducts can be plainly recognized on both sides; whether the typical posterior enlargement was present cannot be determined.

3. Embryo no. 7-4. Conditions of the experiment same as in no. 7-3.

Result: Gonads of about the same length on both sides, 4.5 mm. Left gonad somewhat broader than the right and unlike a testis as to surface texture. Left müllerian duct of the female type; right duct shortened as in the normal female.

4. Embryo no. 2-5 (fig. 28). Operated June 21, 1917. Piece of ovary from a young hen grafted on a seven-day-old embryo. Opened, July 2nd. Age of embryo, 18 days; duration of graft, 11 days.

Result: Right gonad, 4.2 mm. long; left gonad, 5.4 mm. Peculiar black pigmentation over most of the surface of the left gonad, thicker over the anterior end. Left müllerian duct present; traces of middle and posterior portions of the right duct.

5. Embryo no. 17-1 (figs. 29, 30). Operated November 19, 1917. Piece of ovary from a month-old chick grafted on a thirteen-day-old embryo. Opened November 26th. Age of embryo, 20 days; duration of graft, 7 days.

Result: Gonads of the male type, but the right gonad very much smaller than the left one; right gonad, 2.6 mm. long; left gonad, 4.5 mm. long. Left gonad markedly pigmented, except over its posterior end. Wolffian ducts rather of the female type; only posterior portions of the müllerian ducts found.

6. Embryo no. 17-5 (fig. 31). Conditions of the experiment same as in preceding case.

Result: Very similar to no. 17-1, but size difference between the gonads not so great. Right gonad, 4.2 mm.; left gonad, 5.3 mm. Over half of the left gonad pigmented. Wolffian ducts of the male type. Left müllerian duct not well developed; slight trace of the right duct.

7. Embryo no. 29-7. Operated May 31, 1918. Piece of ovary from a ten-day chick implanted on an embryo of eleven days. Opened June 9th. Age of embryo, 20 days; duration of graft, 9 days.

Result: Gonads of the male type; right gonad, 4.5 mm. long; left, 5.5 mm. long. The size difference between the two gonads is greater than in the case of normal testes. Wolffian ducts of the male type. Traces of the degenerating müllerian ducts on both sides.



8. Embryo no. 18-9 (fig. 32). Operated November 23, 1917. Piece of ovary from a young hen grafted on an embryo of eight days. Opened December 4th. Age of embryo, 19 days; duration of graft, 11 days.

Result: Gonads of the male type in general; left one larger than the right; left gonad, 5 x 1.5 mm.; right gonad, 3.8 x 1 mm. Surface of the left gonad rough in texture, differing from that of the normal testis. Wolffian ducts of the male type; slight traces of the posterior portions of the müllerian ducts present on both sides.

9. Embryo no. 29-5 (fig. 35). Conditions of the experiment same as for no. 29-7. Age of embryo, 19 days; duration of graft, 8 days.

Result: Gonads of the female type, but the right gonad much elongated in form and larger than the normal right ovary; left gonad, 4.2 x 1.6 mm.; right gonad, 3 x 0.4 mm. Wolffian ducts more or less of the male type. Müllerian ducts developed on both sides; the posterior part of the right duct more developed than is the case in the normal female condition.

10. Embryo no. 1-7. Operated June 14, 1917. Piece of adult ovary grafted on an eight-day embryo. Opened June 24th. Age of embryo, 18 days; duration of graft, 10 days.

Result: Gonads of the female type; left gonad shortened and of irregular shape; right gonad elongated; left gonad 5; right gonad 3 mm. in length. Wolffian ducts rather of the male type; müllerian ducts developed to about the same degree on both sides.

11. Embryo no. 30-6 (figs. 33, 34). Operated June 5, 1918. Piece of ovary about 2 mm. square from a week-old chick grafted on a nine-day-old embryo. Opened June 14th. Age of embryo, 18 days; duration of graft, 9 days.

Result: Gonads of the female type, the right gonad remarkably slender; left gonad, 4.5 mm.; right gonad, 4 mm. in length. Wolffian and müllerian ducts of the female type.

12. Embryo no. 17-4 (fig. 36). Operated November 19, 1917. Whole ovary of a thirteen-day-old embryo grafted on a thirteen-day-old embryo. Opened November 26th. Age of embryo, 20 days; duration of graft, 7 days.

Result: Left gonad nearly like a normal ovary; right gonad does not resemble normal degenerating right ovary, but is more or less like a male gonad; left gonad, 6 x 2.3 mm.; right gonad, 2.5 x 1 mm. Wolffian and müllerian ducts of the female type.

13. Embryo no. 44-16. Operated April 5, 1919. Piece of ovary from young hen (five and one-half months old) grafted on a ten-day-old embryo. Opened April 15th. Age of embryo, 20 days; duration of graft, 10 days.

Result: Gonads of the female type, the right gonad larger than normal; left gonad, 5.5 x 1.6 mm.; right gonad, 3 mm. Wolffian and müllerian ducts of the female type, but the right müllerian duct better developed than in the normal female.

14. Embryo no. 30-9. Experimental conditions the same as in no. 30-6. Age of embryo, 19 days; duration of graft, 10 days. The graft grew very well; its size, 6.5 x 7.0 mm.

Result: Gonads of the female type in general, but smooth in texture similar to testes; shape also similar to that of testes; left gonad, 4.8 x 1.1 mm.; right gonad, 3.5 x 0.7 mm. Wolffian and müllerian ducts nearly as in the normal female.

*d. Summary of intersexual characters produced by gonad grafts.* The intersexual characters produced in chick embryos by the implantation of grafts of gonad tissue upon their chorio-allantoic membranes may now be summarized.

1. In affected embryos having gonads of the male type, the difference in length between the two gonads is greater than is the case with normal testes. The difference between right and left testes in normal embryos does not exceed 0.5 mm. (table 5). In several intersexual embryos, however, the difference in length of the two gonads exceeds 1 mm., and in extreme cases is still greater, as 1.8 mm. in embryo no. 10-5, 1.9 mm. in embryo no. 17-1, and 2.3 mm. in embryo no. 15-5. This length difference between the two gonads is abnormal for the male, but is characteristic of the female, where the left ovary is much larger than the right one. In this regard, then, the affected embryos may be considered as intermediate between normal males and females.

2. In several affected embryos, male-type gonads exist simultaneously with female-type müllerian ducts. Examples of this

condition are: embryos nos. 27-6, 31-3, 47-10, 43-3, among those grafted with testis, and nos. 27-4, 7-3, and 7-4, among those grafted with ovary. This condition represents a mixture of the characters of the two sexes.

3. It is important to note that the intersexual condition described under 2, where male-type gonads are combined with female-type müllerian ducts differs according to the kind of gonad grafted on the embryo in question. In the case of testis grafts, these intersexual embryos have gonads of the male type; that is to say, the two gonads are nearly of the same size. Examples: embryos nos. 27-6, 31-3, 47-10, 43-3, 27-5. On the other hand, such intersexual embryos when produced by ovary grafts have gonads which resemble ovaries; that is, the left gonad is considerably larger than the right one. Examples: embryos nos. 27-4, 7-3, 2-5. Not only are the gonads of the intersexual embryos different in size in such cases, but the shape and texture of the left ovary differs from that of the right, bearing a greater resemblance to the normal left ovary. The condition of the müllerian ducts in the intersexual embryos is also related to the kind of gonad which was responsible for the intersexual condition. Where the grafted gonad was a testis, the müllerian ducts of the affected embryos are undifferentiated (nos. 47-10, 43-3) and show signs of degeneration (as in nos. 27-6, 31-3, 27-5). Intersexual embryos produced by ovary grafts, on the other hand, retain the müllerian ducts, degenerative changes in the ducts are absent, and the left duct is better developed and more differentiated than the right one (nos. 27-4, 7-4, 2-5).

4. The right gonad of female-type embryos is larger than that of the normal female. The right ovary normally develops to a length of 1.5 to 2 mm., and then degenerates and disappears (table 4). On the other hand, in several of the affected embryos, the right gonad measures more than 3 mm., and in a few cases even more, as 3.3 mm. in embryo no. 17-7, 3.5 mm. in embryo no. 30-9, and 4 mm. in embryo no. 30-6. The right gonad in these embryos has a definite outline as compared with the fading outlines of the normal degenerating right ovary, and it resembles a rudimentary or degenerating testis.



5. The process of degeneration of the right müllerian duct is inhibited. In several cases the developed duct persists on the right as well as on the left side. Examples: embryos with testis grafts, nos. 31-3, 47-10, 43-3; embryos with ovary grafts, nos. 27-4, 7-3, 29-5, 1-7, 44-16.

6. The wolffian ducts typical of one sex are associated with the gonads or müllerian ducts typical of the opposite sex. This point is not, however, so striking as the other points.

7. A black pigmentation often appears on the male-type gonads. This is shown in embryos nos. 27-6, 43-3, 2-5, 17-1, and 17-5. It occurs with both testis and ovary grafts. Histological examination of such gonads reveals that the pigment granules occur not only on the surface, but extend into the interior of the organ and are mingled with the tissue of the stroma.

8. The histological changes in the affected gonads will be described in detail in a later paper, and hence will be but briefly summarized at this point. Histological examination shows that:

a. The male-type gonads are structurally modified testes. The tunica albuginea is abnormal and in some cases partially absent. Where present the connective tissue of which it is composed is much looser than in the normal albuginea. The stroma between the seminiferous tubules is also much less compact than in the normal testis. The seminiferous tubules are fewer in number and more irregular in arrangement than in normal testes. Within the tubules the germ cells are reduced in number and inactive or degenerating, as indicated by their staining behavior.

b. The female-type gonads are structurally modified ovaries. They are less modified from the normal than in the case of male-type gonads. In some cases a partial disappearance of the cortical layer was found; in others a degenerating or necrotic condition of the cords of second proliferation was noticeable.

c. The müllerian ducts which are associated with male-type gonads are very similar in structure to the developed ducts of normal females.

## DISCUSSION

*a. Action of sex hormones.* The experiments presented in this paper show that the primary sexual characters of the chick embryo can be altered in several different ways through the implantation of testis and ovary tissue on the membranes of the embryo. Various degrees of intersexuality were produced. Whenever affected embryos resulted, it was always found that the grafts were growing (or had grown) and that a vascular connection had been established between the graft and the embryonic circulation. It is therefore not only a reasonable, but an unavoidable conclusion that the effect produced by the grafts on the embryo must have occurred by way of the circulation. It may indeed be conclusively stated that the grafts produced substances which were carried to the embryo in the blood stream and modified its sexual differentiation.

It may therefore be regarded as demonstrated that the gonads secrete substances which may be designated sex-hormones, which control or modify both primary and secondary sexual characters. The experimental work presented in this paper is in complete accord with Lillie's explanation of the free-martin condition in cattle, namely, that the free-martin is a female whose primary sexual characters have been modified in the male direction by the action of hormones originating in the testes of the male cotwin. My work shows that testicular and ovarian secretions actually do have such modifying effects on the primary sexual characters.

*b. Specific function.* It is necessary to determine whether or not the modifications observed in these experiments are a specific reaction to gonad secretions or whether they might not be produced in response to secretions from other organs. This point has been subjected to extensive experimental tests with the following results.

Pieces of organs other than gonads were grafted on chick embryos in the same manner as in the case of gonads. The organs used were: thymus, thyroid, spleen, liver, kidney, and some others. Experiments of a similar kind have also been performed in this laboratory by Mr. B. H. Willier, and I had the

privilege of examining Mr. Willier's material; I am indebted to him for his kindness in this matter. From my own material and that of Mr. Willier there were available the following embryos: twelve embryos from thymus grafts; eight embryos from thyroid grafts; two embryos from thyroid and thymus grafts, i.e., pieces of both organs implanted on the same embryo; nine embryos from spleen grafts; three embryos from kidney grafts, and two from liver grafts. It is understood that in all of these cases the grafts grew.

In spite of the fact that grafts from these organs grow more readily and faster and attain a larger size than grafts from gonads, in no case was any effect produced upon the urinogenital systems of the embryos which had borne the grafts.<sup>2</sup> All of the embryos thus obtained are normal males or females, as regards their reproductive systems. I do not wish to imply that these grafts may not have produced some effects upon the embryos, but it may be affirmed with certainty that they had no effect upon the sexual differentiation.

In view of these facts, it may be conclusively stated that the action of the secretions of the gonads upon the primary sexual characters is specific. Only gonad secretions are capable of modifying the sexual characters in experiments of this kind.

*c. Effect of duration of grafts.* An examination of the relation between the duration of the graft and the extent of modification of the embryos will give us some idea of the length of time during which the sex-hormones must act in order to produce an effect. The duration of the graft, that is, the length of time which has elapsed between operation and examination, is a rough measure of hormone dosage employed in each case; the longer the duration, the greater the dosage, presumably. Other factors must, however, be considered. Chief among these is the degree of growth of the graft. Although the same procedure was followed in each operation, and approximately the same amount of tissue implanted, the mode and degree of growth of the grafts

<sup>2</sup> In two cases, however, one with spleen graft and one with liver graft, the left testis showed the same black pigmentation as in the case of several gonad grafts.



was very variable. One graft would grow readily; while another would become partly necrotic. Another factor to be considered is the condition of the embryo on which the graft is implanted. Even if the grafts grow equally well and remain upon the embryos an equal length of time, they will not necessarily produce the same degree of effect, since the embryos themselves vary as to age, and presumably also in general metabolic condition, degree of resistance to foreign tissues, and so on. In consequence of these variable factors, it is difficult to determine in experiments of this kind the relation between amount of dosage and degree of modification of the embryos. In general, however, it may be said that grafts must remain upon the embryo for a certain minimum length of time in order to bring about a modification of its sexual characters. This minimum length of time is about one week.

*d. Effect of age of birds furnishing the grafted gonads.* Another point of interest which arises in connection with these experiments is whether the age of the individuals from which the gonads to be grafted were taken bears any relation to the degree of modification produced. The age of the birds whose gonads were removed for grafting varied in these experiments from embryos eight or nine days in age up to adult fowls. It has seemed to me, however, that in many cases, gonads obtained from young chicks, between the ages of one week and one month, were more effective in producing modifications in the embryos on which they were grafted than gonads from birds of other ages.

*e. Effect of the age of the embryo at the time of grafting.* The experiments yielded definite results upon this point. As has already been stated in the section dealing with the growth of the grafts, the best results were obtained when the grafts were implanted on embryos during the second week of incubation; that is, from the seventh to the thirteenth day. The reason for this fact is not difficult to find. In the chick embryo the differentiation of the sexes appears on about the sixth or seventh day of incubation and progresses rapidly to the eleventh and twelfth days. This period, approximately the second week of incubation, is then the period of active sexual differentiation. It is char-

acterized by the differentiation of the seminiferous tubules in the male and the cortical cords in the female, by the retrogression of the müllerian ducts in the male and the degeneration of the right ovary and duct, and differentiation of the left duct into an oviduct in the female.

As a consequence of the developmental time relations with reference to sexual differentiation, it is reasonable to expect that the grafts will produce the greatest effect during the second week of incubation. This period is the period of sexual differentiation; the sexual characters have not yet become fixed, and hence it is reasonable to suppose that they may be controlled by introduced factors. It is probable that the grafts would be still more effective if they could be implanted before sexual differentiation begins, that is, on the fourth or fifth day; but as already stated, grafts do not grow on such early embryos, owing to the fact that the allantoic circulation has not yet been established.

*f. Degree of reversibility.* Examples of natural or experimental reversals of sex have already been quoted. Such cases are those of the free-martin (Lillie), the guinea-pig and rat (Steinach, Moore), Bonellia (Baltzer), and Crepidula (Gould). Intersexes or sex intergrades have been obtained in pigeons by Riddle, in the gypsy-moth by Goldschmidt, in *Simocephalus* by Banta ('16 a, b), and in *Drosophila* by Sturtevant ('20). In these cases various degrees of reversal of sex were noted, and this reversal was correlated in a quantitative way with the degree of development of the sex organs. The cases of Lillie, Steinach, and Moore are the only cases in which the sex reversal was explained as the direct result of the action of sex-hormones produced by the gonads.

The present experiments bear upon the problem of the reversibility of sex. Intersexual conditions were produced in chick embryos by grafting pieces of testis and ovary upon their membranes. The interpretation of the intersexual individuals is rendered very difficult in the case of experiments of this kind, since we do not with certainty know the sex of the embryos on which the grafts were implanted. In other words, we do not

know what sex the embryo would have been if allowed to develop without experimental interference. Nevertheless, examination of these embryos permits us to draw with reasonable certainty some conclusions concerning their original sex and the degree to which this sex had been modified in the direction of the opposite sex.

It is highly probable that certain of these embryos which received testis grafts were originally females and were subsequently modified in the male direction through the presence of the testis graft. This appears to be the case in embryos nos. 27-6, 31-3, 47-10, 43-3, 27-5. Certain structural features of these embryos indicate that they had originally developed and differentiated to a certain extent in the direction of femaleness. This is evidenced principally by the presence and degree of differentiation of the müllerian ducts; both conditions are similar to those of the female. It is difficult—in fact, impossible—to account for the condition of the müllerian ducts in these embryos except on the basis that the embryos were originally females and had differentiated to this extent in the female direction. There is no possibility that the presence of testis grafts on such embryos could have induced the differentiation of the müllerian ducts. Hence we are compelled to believe that these embryos were originally females. But they possess gonads of the male type. There is no escape from the conclusion that their gonads, originally ovaries, have been greatly modified in the male direction through the action of the secretion from the testes grafted on their membranes.

Certain other embryos of the female type show modification in the male direction, as embryos nos. 17-7, 22-13, 22-9. In these cases the embryos were eleven and thirteen days old when the testis graft was implanted on them. They had therefore attained considerable differentiation in the female direction before the testis hormone acted upon them. In these cases the right ovary persisted—a result which must be ascribed to the action of the testis hormone. We may say that the testis secretion cannot cause the disappearance of the right ovary, or, to put the matter in another way, it tends to make both gonads develop equally.



In the case of ovary grafts, certain embryos were obtained which were probably originally males and which have been modified in the female direction. Such cases are nos. 27-4 and 2-5. These embryos have gonads of the male type, but the left gonad is considerably larger than the right one. We may conclude that these gonads began to develop in the male direction, but after coming under the influence of the ovary graft developed in the female direction. The müllerian ducts of these embryos also show a modification in the female direction; the left duct is better developed and more differentiated than the right one. The left duct does not, moreover, show any signs of degeneration. These conditions can again be interpreted as the consequence of the action of an ovarian secretion upon an originally male embryo. Similar conditions are exhibited by embryos nos. 17-1, 17-5, and 18-9, which also received ovary grafts. These embryos have gonads of the male type, but the left one is again larger than the right one and there are no müllerian ducts. These embryos may be regarded as originally male embryos which were less markedly modified in the female direction than is the case with the two embryos just discussed, nos. 27-4, and 2-5. The reason for this becomes apparent when the experimental conditions in these cases are considered. Grafts were made on embryos nos. 17-1, 17-5, and 18-9 when they were eleven and thirteen days old. Hence they had already undergone considerable differentiation in the male direction, and it is not to be expected that the ovarian secretion could produce such marked effects at that late stage of development as in the cases of nos. 27-4 and 2-5, where the grafts were implanted at nine and seven days, respectively.

Female-type embryos nos. 30-6, 29-5, 1-7, 17-4, 30-9, and 44-16, on which ovary had been grafted, are somewhat puzzling. These embryos have a persistent right gonad. It is difficult to say whether these embryos were originally male and have been markedly transformed in the female direction or whether they were originally females in which the gonads have been stimulated to supernormal development by an excess of ovarian secretion.

In several male-type embryos—nos. 27-6, 43-3, 2-5, 17-1, 17-5, and 44-2—a black pigmentation was present in the gonads. It is a curious and interesting fact that in every case except no. 27-6, the black pigmentation is on the left gonad, and in every case it is on the anterior portion of the gonad. In number 27-6 the right gonad is also pigmented, but to a much less degree than the left one. I am unable to suggest any explanation of the significance of the pigmentation. Normal testes and ovaries are, of course, never pigmented. Bond ('14) and Pezard ('18) described the occurrence of pigmentation in the ovary of the pheasant in correlation with degenerating processes. In two cases of non-gonad grafts, one case with spleen and the other with liver, I found an exactly similar pigmentation of the left testis. It would therefore appear that this occurrence is not a specific effect of the sex hormones.

Although I have not a large number of cases and although the analysis of the results must in the nature of the experiments be largely of an inferential kind, I believe that these results furnish strong evidence that the sexual characters are reversible and that these characters after having differentiated to a certain extent in the direction of one sex may be altered and modified in the direction of the opposite sex.

*g. Conclusion.* Consideration of the experimental data leads us to the following conclusion. The testis and ovary of the chick secrete certain physiological substances, which we may designate sex-hormones. When these secretions are introduced into the body of an embryo they exercise a specific effect upon its reproductive system. The development and differentiation of one sex is stimulated by the secretion of the gonad of the same sex and inhibited by the secretion of the gonad of the opposite sex. By means of these secretions, the differentiation of sex in the chick can be controlled to some extent. It may furthermore be stated that the two sexes bear a quantitative and not a qualitative relation to each other.

## SUMMARY

1. The purpose of this investigation was to determine whether or not sexual differentiation is controllable and reversible through the action of sex-hormones.

2. The common fowl was used as material.

3. A small piece of testis or ovary was grafted on the chorio-allantoic membrane of a developing chick. Testes and ovaries used for grafting were taken from birds varying in age from an eight-day-old embryo to the adult. The embryos upon which the grafts were implanted varied in age from two to sixteen days at the time of the operation.

4. In a number of cases the grafts grew and established vascular connections with the allantoic blood-vessels.

5. A certain number of the embryos upon which the gonad grafts had proved successful exhibited a greater or less degree of modification of their reproductive systems. The most important alterations were these:

*a.* The simultaneous existence of gonads of the male type and differentiated müllerian ducts of the female type.

*b.* An alteration of the normal size ratio of the two testes. Whereas the two testes in the normal male are of the same size at hatching, in many of these cases the left testis was markedly larger than the right one.

*c.* Persistence of the right gonad in female-type embryos. The right gonad disappears in normal female embryos.

6. In normal chicks it was found that the wolffian ducts are persistent in the female.

7. Grafts of liver, spleen, thyroid, thymus, and other organs on chick embryos produce absolutely no modifications of the reproductive system.

8. The results demonstrate that the testis and ovary produce secretions which have definite and specific physiological functions and which are capable of modifying the primary sexual characters.

9. It is shown by these experiments that sexual differentiation may be reversed in the chick. This result supports the explanation advanced by Lillie to account for the production of the free-martin in cattle.



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## PLATE 1

### EXPLANATION OF FIGURES

- 1 Example of a graft growing on the chorio-allantoic membrane, showing also the vascular connections. Ovary graft; embryo no. 17-5. *Gr*, graft; *bv*, blood-vessel.  $\times 3$ .
- 2 Section of the same graft. *Fo*, follicles. Oc.  $2 \times$  obj. 1/12.
- 3 Section of normal adult ovary. *Fo*, follicles. Oc.  $2 \times$  obj. 1/12.

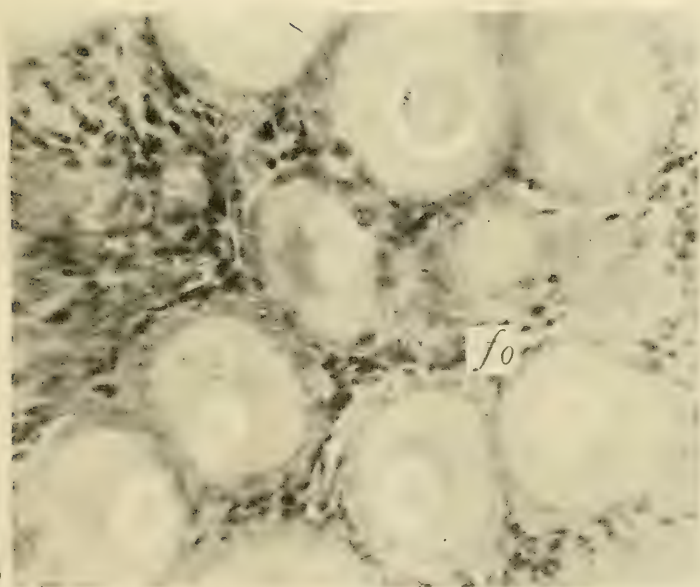




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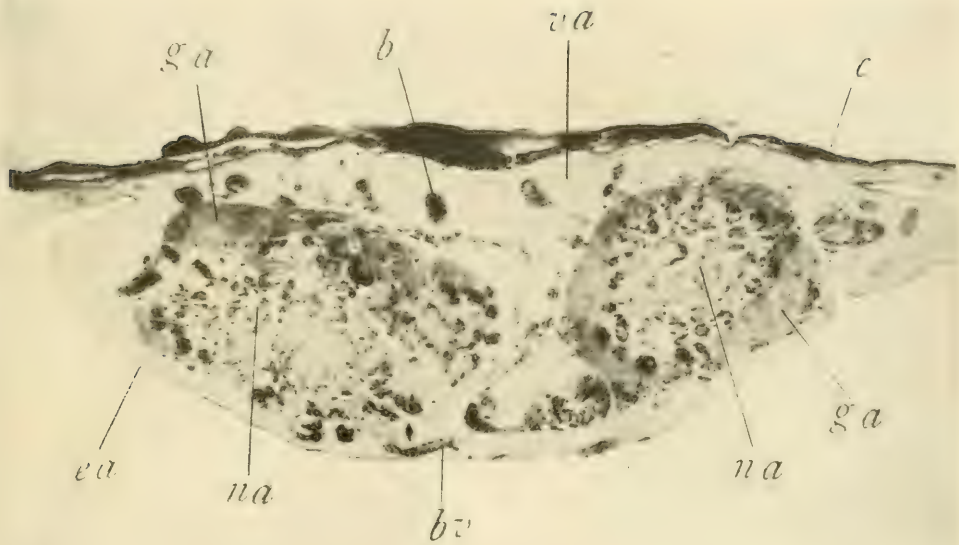
## PLATE 2

### EXPLANATION OF FIGURES

4 Example of a growing graft. Testis graft; embryo no. 17-7. *Gr*, graft; *bv*, blood vessel.  $\times 4$ .

5 Section of a testis graft. Embryo no. 20-1. *Ec*, ectoderm of chorion; *ea*, entoderm of allantois; *bv*, blood-vessel; *va*, vascular area; *ga*, growing area; *na*, necrotic area. Oc.  $4 \times$  obj. 32.

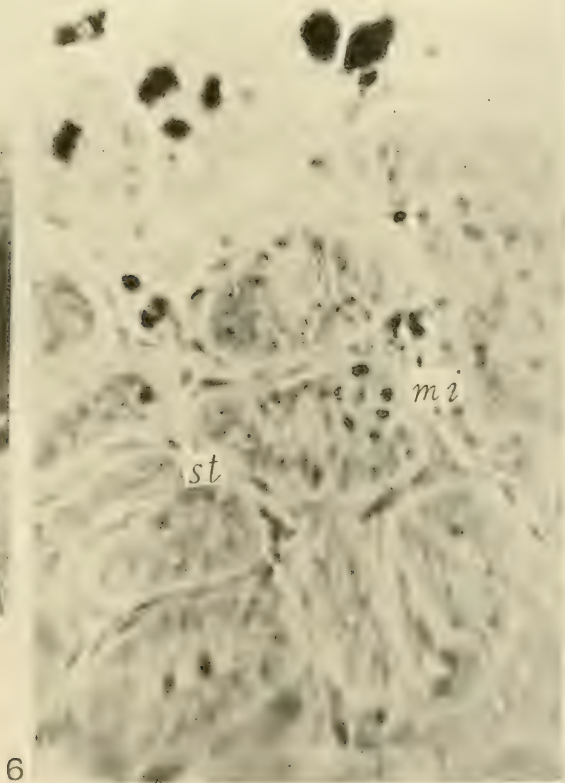
6 Portion of a growing area of the same section, magnified. *St*, seminiferous tubules; *mi*, mitotic figures. Oc.  $2 \times$  obj. 1/12.



5



4



6



### PLATE 3

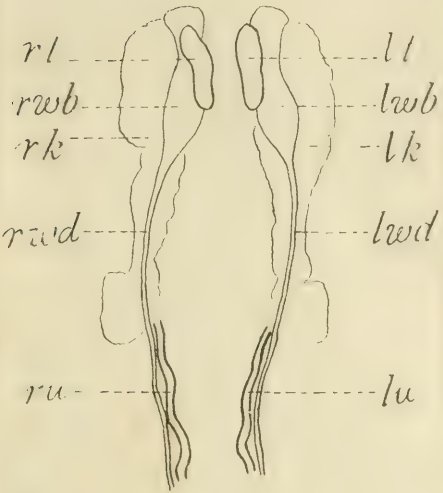
#### EXPLANATION OF FIGURES

7 Urinogenital system of a normal male embryo, eighteen days old. *Rt*, *lt*, right and left testis; *rw*, *lw*, right and left wolffian bodies; *rvd*, *lwd*, right and left wolffian ducts; *ru*, *lu*, right and left ureters; *rk*, *lk*, right and left kidney.  $\times 4$ .

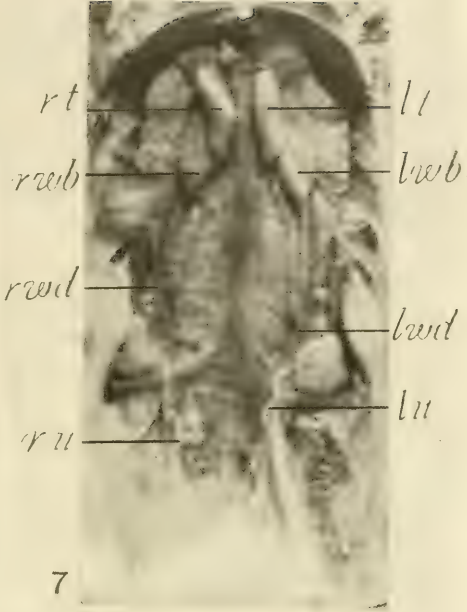
8 Diagram of the urinogenital system of the same embryo.

9 Urinogenital systems of a normal female embryo, eighteen days old. *Lo*, left ovary; *rmd*, *lmd*, right and left müllerian ducts; other abbreviations as in figure 7.  $\times 4$ .

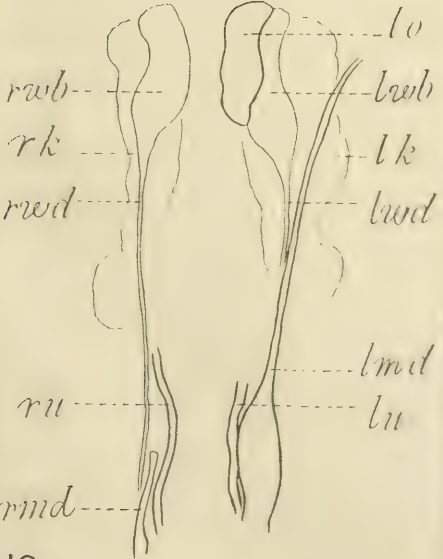
10 Diagram of the same embryo.



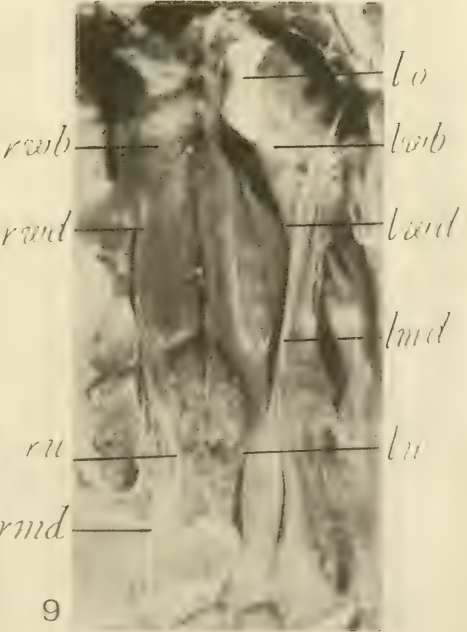
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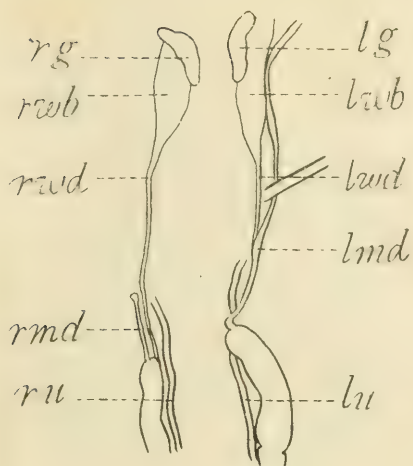
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## PLATE 4

### EXPLANATION OF FIGURES

- 11 Urinogenital system of embryo no. 27-6, modified by testis graft. *Rg, lg*, right and left gonads; *rvb, lwb*, right and left wolffian bodies; *rwd, lwd*, right and left wolffian ducts; *rmd, lmd*, right and left müllerian ducts; *ru, lu*, right and left ureters. Same abbreviations used in all subsequent figures.  $\times 3$ .
- 12 Diagram of the urinogenital system of the same embryo.
- 13 Urinogenital system of embryo no. 31-3, modified by testis graft.  $\times 3$ .
- 14 Diagram of the urinogenital system of the same embryo.

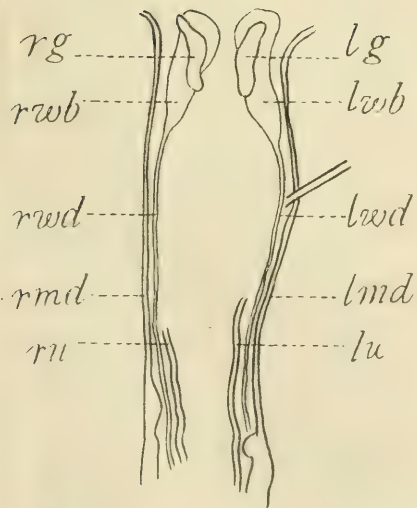




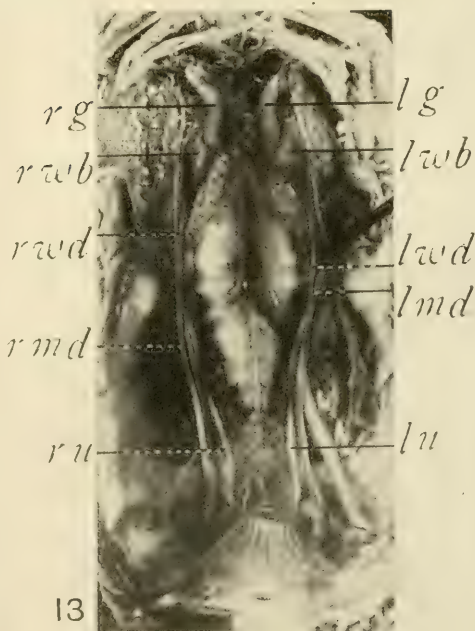
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## PLATE 5

### EXPLANATION OF FIGURES

- 15 Embryo no. 47-10, modified by testis graft.  $\times 3$ .
- 16 Embryo no. 43-3, modified by testis graft.  $\times 4$ .
- 17 Embryo no. 27-5, modified by testis graft.  $\times 3$ .
- 18 Embryo no. 15-5, modified by testis graft.  $\times 3$ .

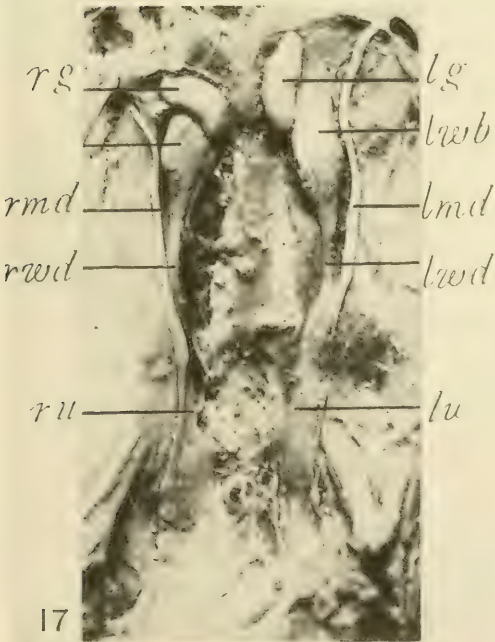
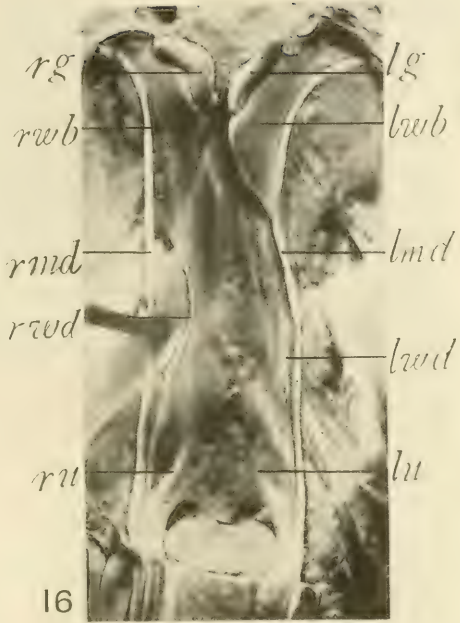
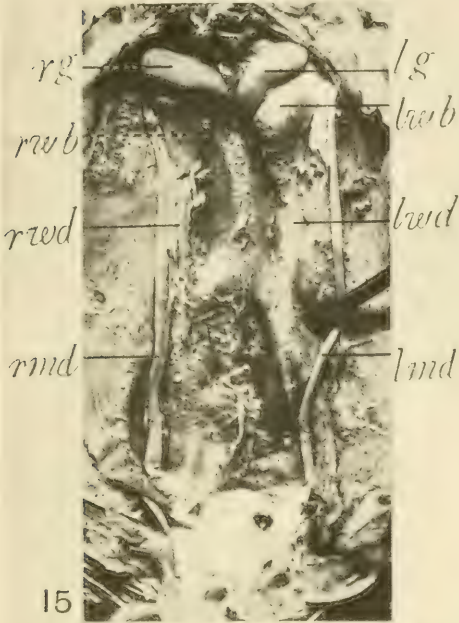
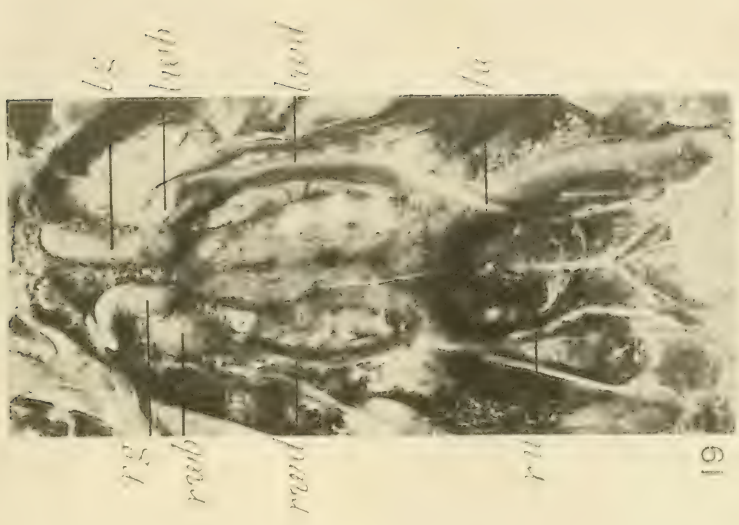
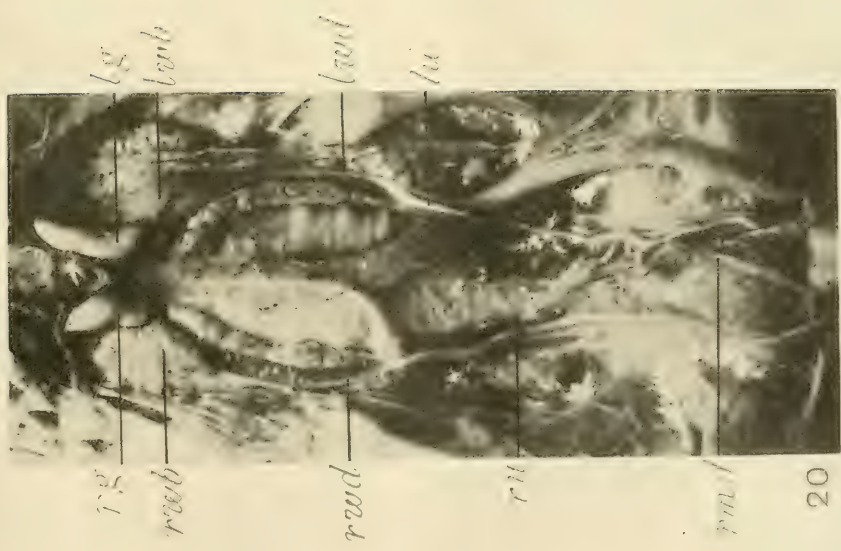




PLATE 6

EXPLANATION OF FIGURES

- 19 Embryo no. 45-6, modified by testis graft.  $\times 4$ .  
20 Embryo no. 45-18, modified by testis graft.  $\times 4$

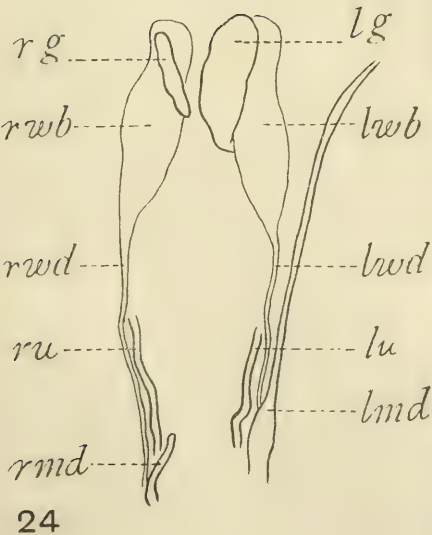
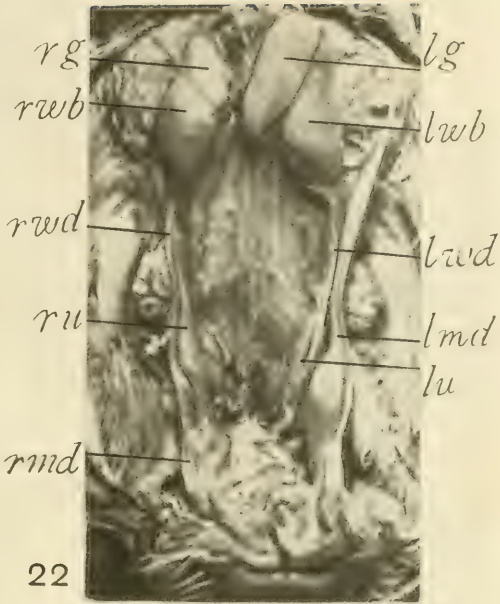


## PLATE 7

### EXPLANATION OF FIGURES

- 21 Embryo no. 8-5, modified by testis graft.  $\times 3$ .
- 22 Embryo no. 22-13, modified by testis graft.  $\times 4$ .
- 23 Embryo no. 17-7, modified by testis graft.  $\times 4$ .
- 24 Diagram of the urinogenital system of the same embryo.

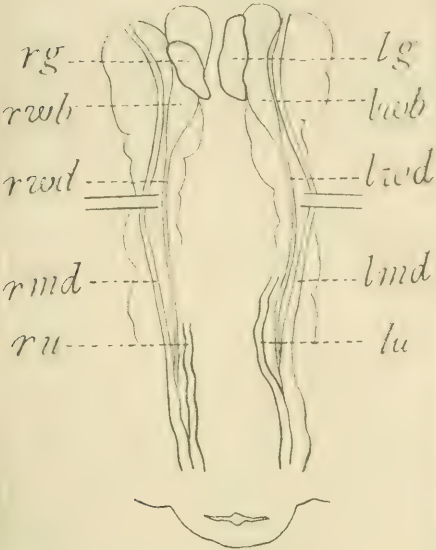




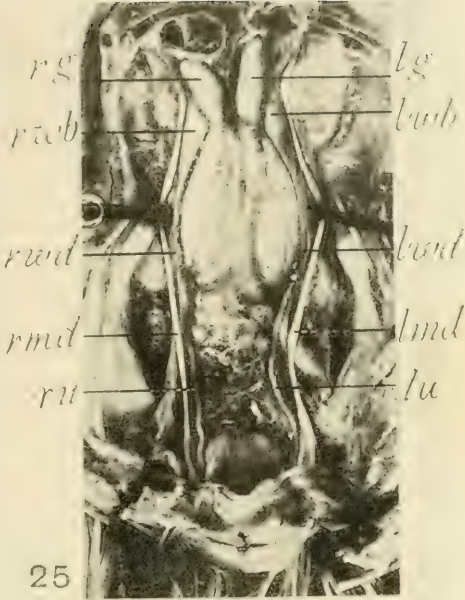
## PLATE 8

### EXPLANATION OF FIGURES

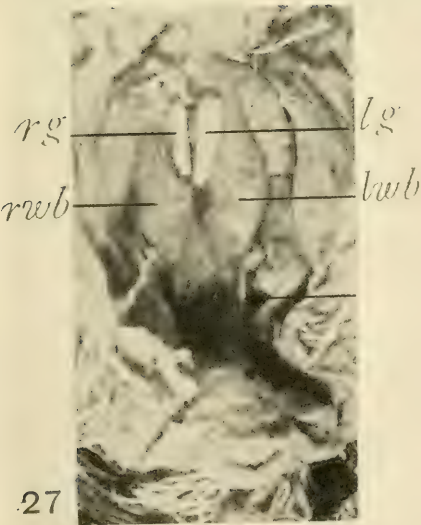
- 25 Embryo no. 27-4, modified by ovary graft.  $\times 3$ .
- 26 Diagram of the urinogenital system of the same embryo.
- 27 Embryo no. 7-3, modified by ovary graft.  $\times 3\frac{1}{2}$ .
- 28 Embryo no. 2-5, modified by ovary graft.  $\times 4$ .



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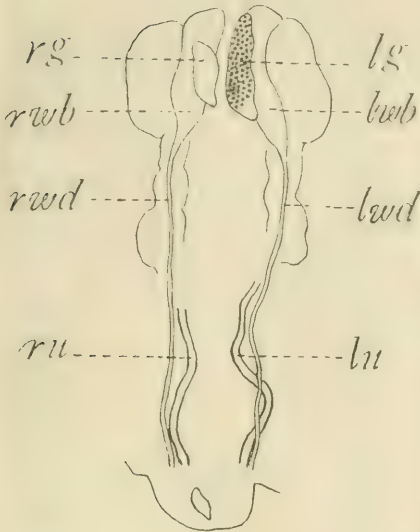
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## PLATE 9

### EXPLANATION OF FIGURES

- 29 Embryo no. 17-1, modified by ovary graft.  $\times 3$ .
- 30 Diagram of the urinogenital system of the same embryo.
- 31 Embryo no. 17-5, modified by ovary graft.  $\times 3$ .
- 32 Embryo no. 18-9, modified by ovary graft.  $\times 3$ .



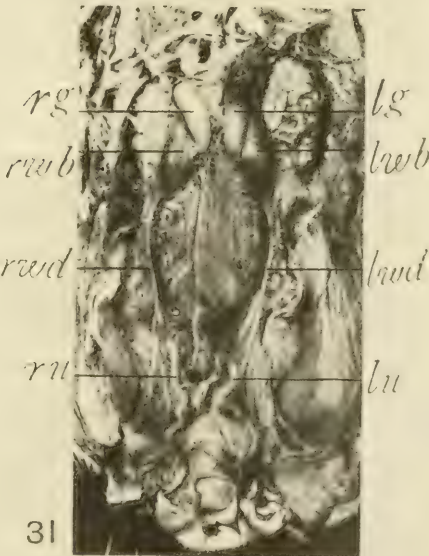
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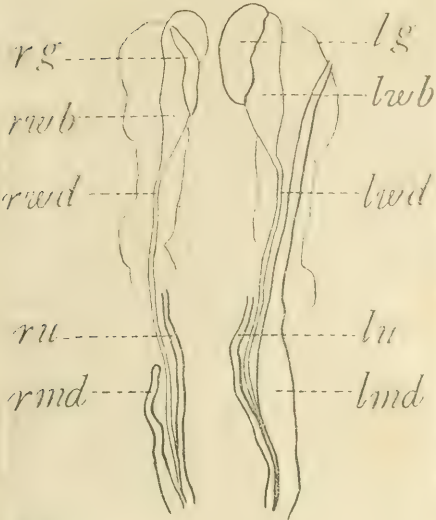


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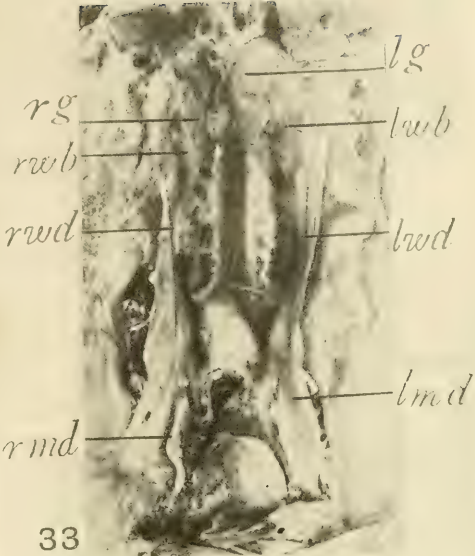
## PLATE 10

### EXPLANATION OF FIGURES

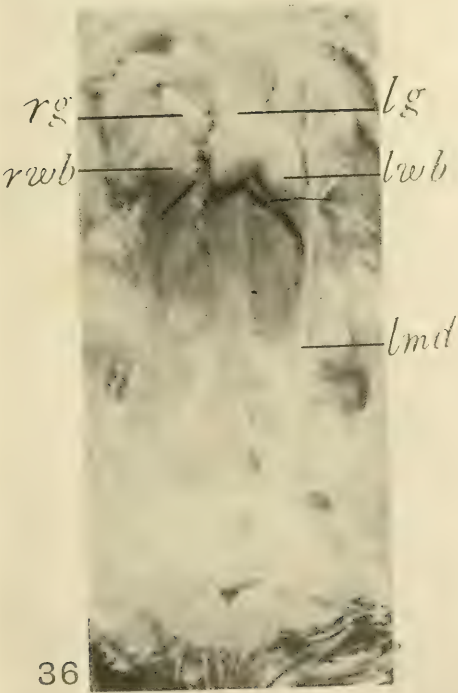
- 33 Embryo no. 30-6, modified by ovary graft.  $\times 3$ .
- 34 Diagram of the urinogenital system of the same embryo.
- 35 Embryo no. 29-5, modified by ovary graft.  $\times 3$ .
- 36 Embryo no. 17-4, modified by ovary graft.  $\times 4$ .



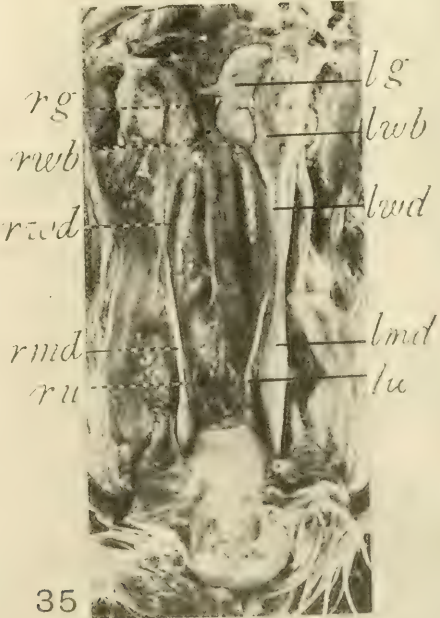
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Resumen por el autor, Benjamin H. Willier,  
Hull Zoological Laboratory, University of Chicago.

### Estructuras y homologías de las gonadas del "free-martin."

En el "free-martin" (hembra estéril gemela de un macho normal, en el ganado vacuno) una gonada indiferente con una determinación primaria femenina puede, bajo la influencia de los hormones sexuales producidos por el macho normal gemelo, presentar varios grados de transformación en la dirección del macho. De las observaciones estructurales obtenidas mediante el estudio de diez y seis casos (siete fetales y nueve post-natales) se desprende que las glándulas reproductoras de los free-martins pueden distribuirse en tres grupos distintos, los cuales pueden caracterizarse como grados bajo, medio y alto de transformación en la dirección del macho, y constituyen por consiguiente, una cadena de eslabones próximos entre un ovario embrionario y el testículo. Todos los órganos de estas gonadas modificadas son afectados. Los cordones sexuales exhiben una serie de gradaciones entre los cordones medulares y los túbulos seminíferos (completa con la excepción de la células sexuales masculinas, que faltan).

La red ovárica se transforma en una red testicular, principalmente mediante el desarrollo de conexiones (túbulos rectos) entre los túbulos de la red y los túbulos seminíferos, y mediante conexiones entre aquellos y los tubulos del epidídimo. En las gonadas menos transformadas el epidídimo falta; en las que exhiben un grado moderado de transformación solamente se presenta la cabeza del epidídimo, y en las gonadas más completamente transformadas existe un epidídimo completo. La distribución de los vasos sanguíneos varía desde la del ovario típico hasta la disposición del testículo típico. De esto se desprende que el órgano sexual más fundamental puede invertirse casi completamente por medio de hormones del sexo opuesto. La diferenciación sexual no está, pues, determinada exclusivamente por la unión de los gametos.

## STRUCTURES AND HOMOLOGIES OF FREE-MARTIN GONADS

BENJAMIN H. WILLIER

*Hull Zoological Laboratory, University of Chicago*

EIGHTEEN FIGURES

### INTRODUCTION

A female which is born co-twin with a normal male in cattle is usually sterile, and is known among stockmen as a 'free-martin.' The internal reproductive organs of such a female are decidedly male-like, and the external genitalia are usually female-like, although they may be modified also in the male direction. Lillie ('17) showed that the sterile free-martin is zygotically a female which is modified in the male direction by the action of sex hormones from the male twin. These hormones circulate in the vascular systems of both foetuses, owing to the establishment of a common circulation by an early fusion of the embryonic membranes and the anastomosis of the extra-embryonic blood-vessels of the two individuals. If no vascular connections between the twins are made, the female is a 'fertile free-martin.'

The effect upon the foetal reproductive glands of the introduction of the hormones from the male embryo into the circulation of the female twin was described by Chapin ('17). The reproductive organs which are present in the indifferent stage at the time when the secretion from the male enters the circulation of the female develop toward the male condition, while those structures which would develop at the time of sex differentiation in the normal female, are absent. That is to say, the tunica albuginea, first set of sex cords,<sup>1</sup> rete testis, and epididymis

<sup>1</sup> The term sex (or sexual) cord is used to include the invaginations of the germinal epithelium, whether they are male or female structures; in the case of the testis there is one set of sexual cords, which are destined to form the func-

develop, while the distinctly female structures, the cords of Pflüger, and definitive ovarian albuginea fail to develop.

In both the foetal and post-natal gonads of free-martins, the tunica albuginea, sex-cord region, and rete are of constant occurrence, although each structure may vary greatly as to size, degree of differentiation, and degree of transformation in the male direction. Such structures as the tubules of the epididymis and the spermatic cord are in some specimens entirely absent. There may be a correlation between this high degree of variation in the structure of the reproductive glands of the free-martin and the time and degree of anastomosis of the blood-vessels of the two blastodermic vesicles. In other words, there may be considerable variability in the time at which the internal secretion enters the circulation of the female twin; variations in quantity of the hormone and in the intensity of its action are also conceivable.

Owing to these male characteristics of the gonad, several investigators have misinterpreted the true sex of the free-martin. Hart ('10) and Magnusson ('18), who described the microscopical anatomy of the reproductive organs of the post-natal free-martin, both reached the conclusion that it is an abnormal male. It was not until the embryological history of these gonads was known that a correct interpretation was possible. With the embryological data and from an examination of a number of post-natal gonads of the free-martin, the conclusion is reached

tional seminiferous tubules, while in the case of the ovary there are two sets of sexual cords; the first set forms the medullary cords, which are destined to degenerate and which are homologous with the seminiferous tubules; the second set forms the cords of Pflüger.

The term sex-cord region is used to include both the sexual cords and the inter-cordal tissue (stroma of connective-tissue fibers between which are the interstitial cells).

The term rete is applied to the network of tubules of the rete testis, of the rete ovarii, and of the modified rete of the free-martin gonad.

The term tunica albuginea is used to designate the connective-tissue capsule of the testis, and the layer of connective tissue between the medullary cords and the cords of Pflüger of the ovary. It is the primary tunica albuginea of the ovary and the definitive tunica albuginea of the testis. In the ovary a second layer of connective-tissue fibers develops between the cords of Pflüger and the germinal epithelium; this is the definitive tunica albuginea of the ovary.

that primarily the gonad is female in structure and that secondarily it is transformed into a male gonad by hormonal action.

Since the embryological evidence is alone the key to the correct explanation, it is the purpose of the present account to interpret the microscopic structure of the post-natal gonad on the basis of its development. The facts presented in this paper were gathered in a study of the gonads of seven foetal free-martins (Miss Chapin's material) ranging in length from 7.5 cm. to 28 cm., and from nine post-natal free-martins ranging in age from five days to three years. Data obtained from a study of the gonads of normal males and females of approximately the same sizes and ages are introduced for comparison. For complete lists of the specimens and tabulated summaries of the microscopical findings, tables 1, 2, and 3 may be consulted. A list of the foetal free-martin gonads studied is given in Miss Chapin's tabulated summary (Chapin, '17, p. 478).

We are deeply indebted to Prof. Leon J. Cole, of the University of Wisconsin, for the reproductive glands from the following post-natal free-martins: H-18, H-46, H-36, H-37, H-42, and H-40 of table 1, for the use of the manuscript, "The anatomy of the urino-genital system of the free-martin," prepared by his student, Mr. J. V. Seids, and for other data not contained in the manuscript. Without these the present work would have been impossible. The remaining specimens are from Professor Lillie's collection. Different fixing agents were employed, the more common ones being formalin, Zenker-acetic, strong Fleming's solution, and Bouin's solution. Heidenhain's iron hematoxylin and Mallory's triple stain were the stains most commonly used.

The study of this problem was undertaken at the suggestion of Prof. Frank R. Lillie, and it gives me great pleasure to acknowledge my deep indebtedness to him for instruction and for kindly advice.



## GENERAL INTERPRETATION OF THE POST-NATAL GONADS

Diagrammatic reconstructions shown in figure 1 summarize the detailed study presented beyond. The gonads of the free-martin may be divided into three groups, characterized, respectively, as low, medium, and high degrees of transformations in the male direction. These three groups are represented in figure 1 (B, C, and D) as graphic reconstructions and constitute a chain of connected links between an embryonic ovary (A) and a testis (E). For comparison similar graphic reconstructions of ovaries (A' and A'') and a testis (E) are introduced. Diagram A represents an ovary in the indifferent stage of development. It is covered with a superficial layer, the germinal epithelium (*ge*), from which the first set of sexual cords (*sc*) arise by invagination. At the anterior end, the rete tubules (*r*) enter the hilum and project for a short distance posteriorly into the sex-cord region (*sc*). The ovarian blood-vessels (*bv*) also enter the gonad at the hilum. Under normal conditions, this gonad (A) differentiates into an ovary, but under the influence of the male sex hormones it differentiates into a gonad which is morphologically a testis.

Diagrams A' and A'' illustrate in two stages the normal differentiation of an ovary from the indifferent stage A. The ovary shown in A' may be regarded as derived from the indifferent stage A by the addition of a second set of sexual cords, the cords of Pflüger (*p*), which arise as secondary proliferations of the germinal epithelium (*ge*). Between the first set of sexual cords (*sc*), which in the ovary are known as medullary cords, and the cords of Pflüger (*p*) is located the primary tunica albuginea (*ta*), homologous with the tunica albuginea (*ta*) of the testis (E). Between the germinal epithelium (*ge*) and the cords of Pflüger (*p*) is the definitive ovarian albuginea (*oa*), which has no homologue in the testis. The rete tubules (*r*) have penetrated to the posterior end of the medullary cord region, their eccentric position is retained, and connections (*tubuli recti*) are never established between the medullary cords (*sc*) and the rete tubules (*r*). The arrangement of the dots in the stippled area (*sc*) of the

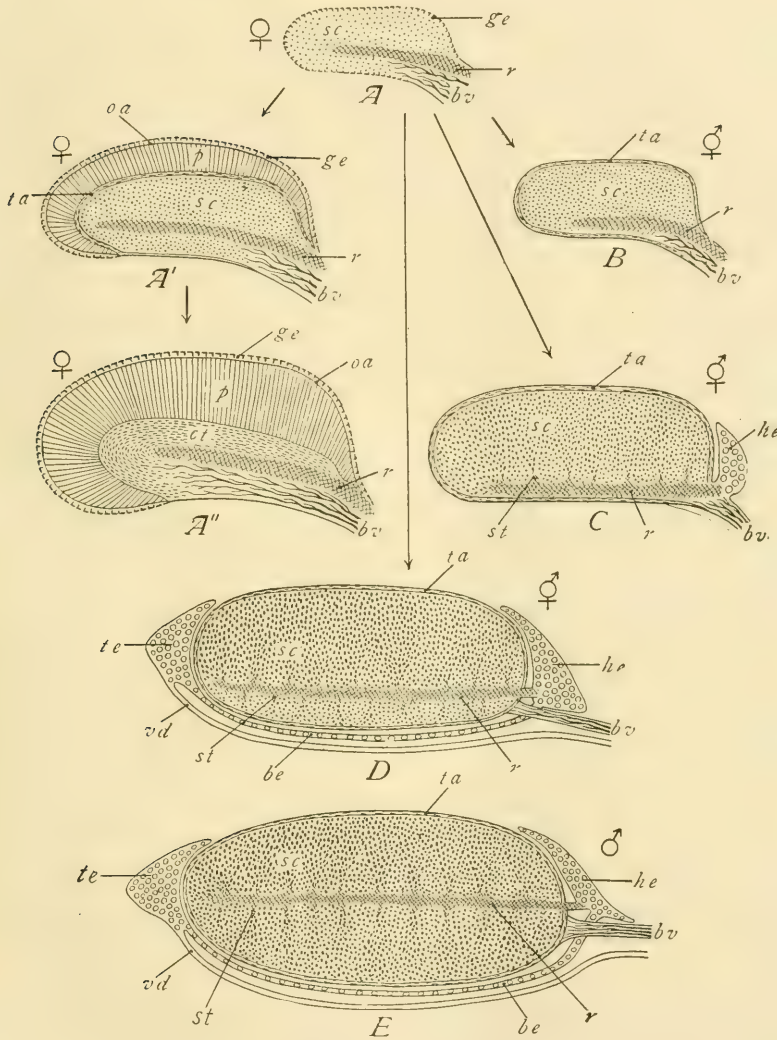


Fig. 1<sup>2</sup> Diagrammatic reconstructions to show a normal testis, and an indifferent stage of an ovary, which under normal conditions differentiates into an ovary (shown in two stages), but under the influence of male sex hormones may form gonads which exhibit three stages of transformation in the male direction.

<sup>2</sup> I am indebted to Mr. Kenji Toda for valuable assistance in the preparation of the drawings.

diagram indicates that the sexual cords bear no definite orientation to the rete (*r*).

Diagram A'' represents an ovary at about the time of birth. It differs from the ovary A' chiefly through the increase in size of the cords of Pflüger (*p*) and the retrogression of the medullary-cord region. In the latter the medullary cords have mostly degenerated; connective-tissue fibers (indicated by the area of short dashes, *ct*) and blood-vessels have increased. The rete, although distinct, is destined to degenerate in still older stages.

Diagrams B, C, and D, on the other hand, illustrate three steps in the transformation of an ovary of the indifferent stage A into a free-martin gonad which is morphologically a testis. Diagram B represents a low degree of transformation in the male direction. The sex-cord region (*sc*) is comparatively small and poorly organized. The arrangement of the dots in the stippled area (*sc*) indicates that the sexual cords are irregularly arranged. The rete (*r*) lies in its primitive position at the hilus, and has not penetrated far into the sex-cord region (*sc*). It is important to note that stage B resembles the indifferent stage A in possessing homologous parts; however, stage B differs from stage A in the absence of the germinal epithelium (*ge*) and the presence of a tunica albuginea (*ta*). It differs from stage A' in the absence of the cortex (cords of Pflüger, definitive albuginea, and germinal epithelium), but resembles it in the irregular arrangement of the sexual cords (*sc*), the absence of connections (tubuli recti) between the sexual cords and the rete tubules, the position of the blood-vessels and rete, and the presence of a tunica albuginea (*ta*).

The next step in transformation is illustrated by diagram C, and exhibits an increase in size and organization of the sex-cord region (*sc*); establishment of connections (tubuli recti, *st*) between the sexual cords (*sc*) and the rete tubules (*r*) on all sides of the rete except the side adjacent to the tunica albuginea (*ta*); and the addition of a head of an epididymis (*he*), the tubules of which are connected with the rete tubules (*r*). The sexual cords in this stage of transformation are so arranged that they radiate out from the straight tubules (*st*), as indicated in the diagram by



the arrangement of the dots in the stippled area (*sc*). Entering the anterior end of the gonad just below the rete is a distinct cord of convoluted blood-vessels, which, aside from its eccentric position, resembles the vascular cord (*bv*) of the normal testis (E).

The highest step in the transformation series is shown in diagram D, which very closely resembles the normal testis (E). By the growth of the sex-cord region, the rete (*r*) has been shifted toward the center of the gonad, so that on all sides the rete tubules make connections (*st*) with the sexual cords (*sc*). Although the rete (*r*) in D is still slightly eccentric in the sex-cord region (*sc*), its position marks a distinct advance toward maleness; the rete normally forms a core in the center of the testis (E). At the anterior end of gonad D, the rete tubules (*r*) make connections with the tubules of the head of the epididymis (*he*). Attached to the posterior end of the gonad is the tail of the epididymis (*te*), connected with the head (*he*) by the body of that organ (*be*). Passing anteriorly from the tail of the epididymis (*te*) is a vas deferens (*vd*). It will be noted that as the rete shifts toward the center of the gonad, the vascular cord (*bv*) also moves in the same direction. In this highly transformed gonad (D) the epididymal structures, vas deferens, and rete are in essentially the same mutual relations as in the normal testis (E).

#### THE MICROSCOPICAL ANATOMY OF THE POST-NATAL GONADS

The following pages comprise detailed descriptions of the microscopical anatomy of the reproductive glands of nine post-natal free-martins, comparisons with the structure of the gonads of foetal free-martins and of normal gonads, and discussions based on these comparisons. An examination of the microscopical anatomy indicates considerable variation in structure. Some have approximately the typical structure of a testis, while others are much less typically male. In other words, they exhibit a graded series of transformations between an ovary and a testis. Three more or less distinct steps may be recognized, which may be characterized as low, medium, and high degrees of transformations in the male direction. An examination of this series



will furnish additional information concerning how complete the transformation of a zygotically determined female into a male individual by the action of male sex hormones is possible. It will also furnish a positive demonstration of the existence of 'sexual dimorphism' in the sex glands of mammals; that is, for the theory that the primordium of the mammalian gonad has the potentialities for the development of both ovary and testis.

*A. Gonads having a low degree of transformation*

The reproductive glands belonging to this group are the least modified of any in the series, yet they show the majority of the typical male structures. They are characterized by their undescended or ovarian position, by the absence of an epididymis, and by the small size and low degree of organization of the sex-cord region. The rete is comparatively large and well differentiated.

The histories of the two specimens which belong to this group are detailed as follows:

Case H-36. Born co-twin to potent bull. A Holstein freemartin aged approximately two and a half years when killed, December 15, 1916. External genitalia typically female. Uterus rudimentary. Small gonads located in normal ovarian position. Rudimentary spermatic cord; poorly developed seminal vesicles enter the vasa deferentia. Only the posterior portion of the vasa deferentia developed.

Case H-40 (fig. 2). Born co-twin to normal bull. Three years old when slaughtered, February 3, 1917. External genitalia female, but smaller than normal. Vagina rudimentary; uterus absent. Wolffian ducts well developed, but made no connections with the gonads. Gonads in ovarian position. A plexus of blood-vessels was attached to the anterior border of each gonad. Seminal vesicles enter vasa deferentia (Wolffian ducts).

*The tunica albuginea and tunica vasculosa.* Owing to poor preservation, the gonad of H-36 will be only briefly described. It is covered with a thick capsule of densely arranged connective-tissue fibers, the tunica albuginea, as in the normal testis. Imme-

diately under the compactly arranged fibers are similar fibers more loosely arranged and enclosing blood-vessels. The position and structure of this vascular zone makes it homologous with the tunica vasculosa of the normal embryonic testis in cattle (fig. 4). Its existence as a distinct layer in the normal foetal testis is lost shortly before birth, through an increase in connective-tissue fibers and its intimate union with the tunica albuginea (table 3 for details). Thus the two layers are merged into one thick layer, known as the tunica albuginea, the inner portion of which contains the blood-vessels. It is thus seen in this free-martin gonad that the embryonic relationships of the tunica albuginea and tunica vasculosa are retained.

Gonad H-40 (fig. 2) the oldest one of the entire series, is undergoing certain pathological changes. Of chief importance is the infiltration of connective-tissue fibers into the sex-cord region, thereby crowding out some of the sexual cords and the interstitial cells. The sex-cord region is approximately reduced to a crescentic area which surrounds the rete region except where the rete comes in contact with the tunica albuginea. The tunica albuginea and the tunica vasculosa are not distinguishable as they are in H-36, but the blood-vessels are scattered throughout the inner portion of the thickened capsule (fig. 2, *bv*). The connective-tissue fibers of the tunica albuginea are very compactly arranged, and this arrangement is maintained and continued on into the sex-cord region, so that no line of demarkation can be recognized between the sex-cord region and the tunica albuginea. Comparatively, the tunica albuginea of this free-martin gonad has overgrown the usual limits of this structure in free-martin sex glands.

*The sex-cord region.* Immediately under the tunica vasculosa of H-36 a faint trace of a sex-cord region may be recognized. It is represented by only a few sexual cords which in structure resemble seminiferous tubules. Each tubule has a slightly thickened connective-tissue wall, within which is a syncytium of supporting epithelial cells (Sertoli cells). Germ cells are entirely absent.

In the dense connective-tissue fibers which have penetrated the sex-cord region of gonad H-40, islands of sexual cords and interstitial cells are frequently found, and occasionally isolated sexual cords embedded in the dense fibers. These isolated sexual cords, the islands of sexual cords and interstitial cells, and the

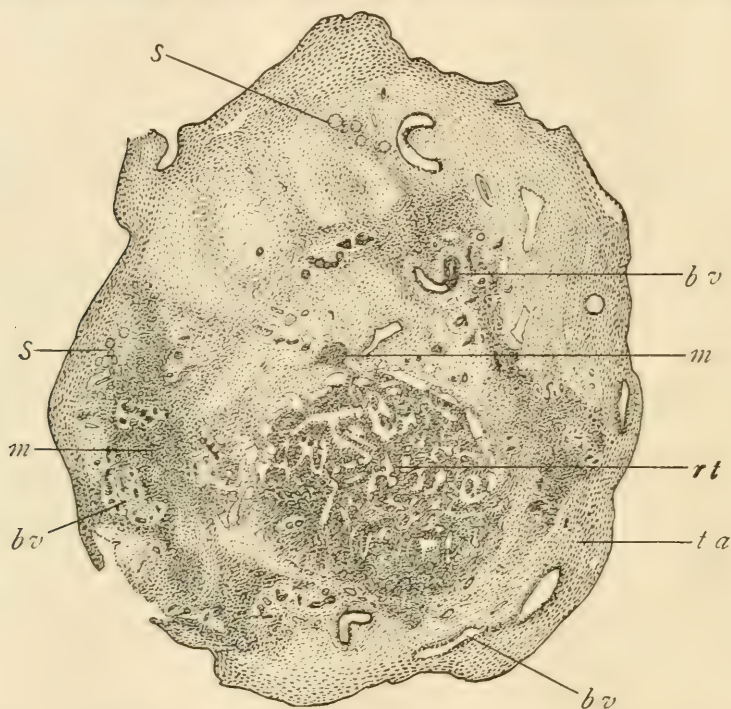


Fig. 2 Transverse section through the middle of free-martin gonad, H-40. *bv*, blood vessels; *m*, areas of interstitial cells and sexual cords; *s*, seminiferous tubules; *rt*, rete tubules; *ta*, tunica albuginea.  $\times 14$ .

crescentic area (mentioned above) constitute the sex-cord region (fig. 2, *s*, *m*). The interstitial cells are abundant, but the sexual cords are comparatively few in number and of small size. The sexual cords are characterized by thickened connective-tissue walls, within which is a syncytium of supporting epithelial cells. This syncytium is free from germ cells. Such a syncytium is formed by the coalescence of a number of supporting epithelial



cells, the cytoplasm of which runs together, but the nuclei remain distinct. The structure of this cell aggregation varies considerably in the different sexual cords. In some the nuclei are closely crowded and irregularly arranged; the amount of cytoplasm varies considerably, for in some cords it is clearly seen, while in others it is hardly detectable. Such an irregular arrangement and crowding of the nuclei resemble the condition in the medullary cords of the normal ovary (N 14, table 2) and of very young seminiferous tubules (N 13, table 3). In other sex cords the supporting epithelium is loose and the nuclei are arranged in a single layer which lines the wall of the cord. The long axes of the ovoid nuclei are arranged perpendicular to the wall of the sex cord, forming a palisade arrangement. From the inner ends of the nuclei cytoplasmic strands extend toward the potential lumen. Such sex cords are typical seminiferous tubules and present a structure similar to the condition in the seminiferous tubules in the testis of a young foetus, with the exception that there are no male germ cells in the free-martin tubules (T 16, T 6, table 3), (fig. 2, *s*). The nuclei of the supporting epithelial cells of both types of sexual cords resemble very closely the nuclei of the Sertoli cells of the normal adult testis, in having a slight amount of chromatin, chiefly distributed in a distinctive nucleolus, and a faintly staining nuclear membrane. A few of the tubules show distinct pathological changes, where the cytoplasm of the supporting epithelial cells has in places disappeared, leaving naked nuclei; in others even the nuclei have disappeared, leaving mere spaces in the dense connective tissue.

*The rete tubules.* Located at the hilus of both gonads is the well-developed cylindrical mass of rete tubules, which constitutes by far the greater portion of the reproductive gland in case H-36. Figure 2, *rt*, shows the primitive position of the rete in H-40 and the way it is marked off sharply from the sex-cord region by connective tissue, so that no connections (tubuli recti) are established between the rete tubules and the sexual cords. No tubuli recti are developed in H-36. The distance of penetration of the rete into the sex-cord region has not been determined in H-36, but in H-40 it only extends two-thirds of the way back.



Morphologically, the rete is a cord of densely arranged connective-tissue fibers in which is a network of anastomosing channels, the rete tubules. These tubules are lined with a single layer of columnar epithelial cells having elongated nuclei. They resemble the rete testis tubules of the normal testis, with the

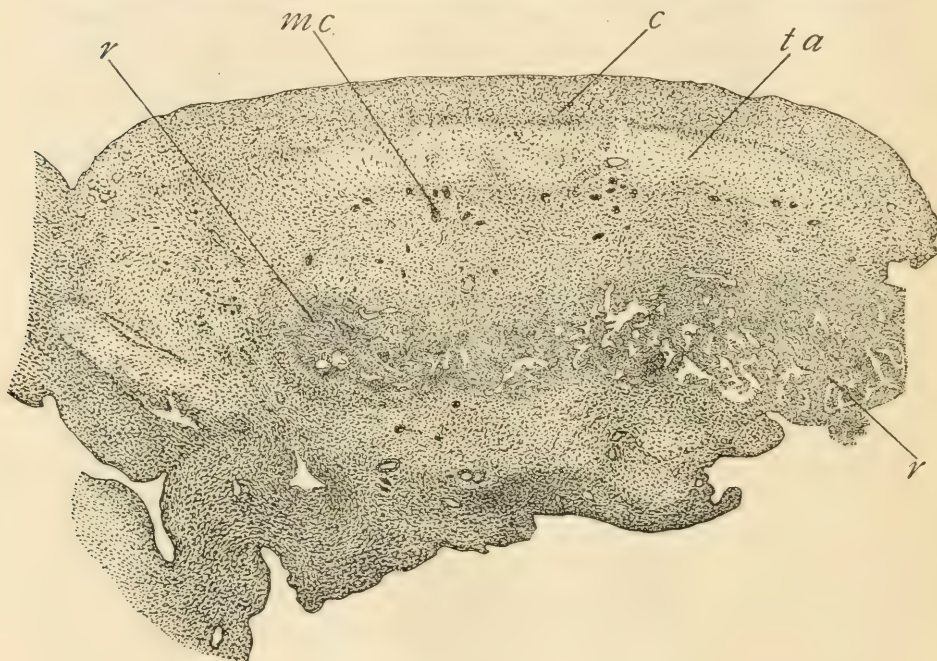


Fig. 3 Longitudinal section through an ovary of a 56-cm. female bos foetus, Bos 8. *c*, cortex; *mc*, medullary cord; *r*, rete enters anterior end and penetrates to posterior end of medullary-cord region; *ta*, tunica primary albuginea between medullary-cord region and cortex.  $\times 21$ .

difference, however, that the epithelial cells of the free-martin tubules are much less regular in arrangement.

The constancy of the eccentric position of the rete region will be understood from the consideration of the normal development of this region in the ovary and the testis. The rete ovarii and the rete testis both originate from a region anterior to the sex gland, and penetrate into the anterior end of the gonad, the point of entrance in each case being the hilum. The rete ovarii

penetrates to the posterior end of the medullary cord region, but not at as early a stage as the rete testis. It retains its primitive position at the hilus of the ovary during its further development (fig. 3). On the other hand, the rete testis penetrates at an

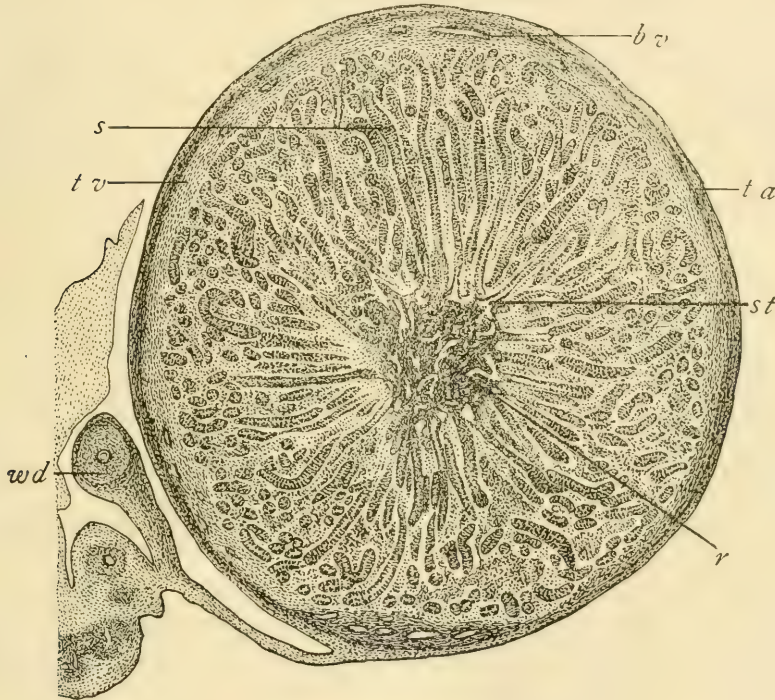


Fig. 4 Transverse section through the middle of the testis of a 20-cm. male *bos foetus*, N 21. *bv*, blood-vessel in tunica vasculosa; *r*, rete testis (note its central position); *s*, seminiferous tubules (note that the seminiferous tubules are more convoluted near the periphery of the testis); *st*, tubuli recti; *ta*, tunica albuginea; *tv*, tunica vasculosa; *wd*, Wolffian duct.  $\times 33$ .

early stage to the posterior tip of the testis, to form an axis from which the seminiferous tubules radiate in all directions (fig. 4). During the early development of the testis the rete lies between the mesentery and the sex-cord region, and as the seminiferous tubules begin to grow, they extend around the sides of the rete until they meet, making the rete appear to be in the center of a round gland; that is, seminiferous tubules grow around the

eccentrically placed mass of rete tubules in such a manner as to enclose it.

Obviously, from the above embryological account, the rete mass is primarily located at the hilus of the free-martin gonad, and since this gonad is transforming into a testis it is to be expected that the rete mass should come to lie in the center of a round gland. However, in gonads having a low degree of transformation in the male direction, as in cases H-40 and H-36, the rete region lies at the hilus. This is due to the small size of the sex-cord region. In other words, the sex-cord region in these cases has not grown enough to surround the rete region.

*B. Gonads exhibiting a medium degree of transformation*

The characteristics of the gonads in this group are: the undescended position which they occupy; the contorted tubules of the epididymis are few or entirely absent (as in H-18); the large size and high degree of organization of the sex-cord region; and the establishment of connections between the rete and the sexual cords, and between the rete and the epididymis.

The histories of the four specimens belonging to this category are as follows:

Case 66. Born co-twin with a normal male calf. Age five days when slaughtered (youngest post-natal free-martin gonad obtained). In place of the uterus were fine ducts; very minute gonads in ovarian position.

Case 42. Born co-twin with normal male. Killed twenty-one days after birth. Uterus reduced in size, vagina short and ending blindly. Gubernaculum absent; gonads in ovarian position.

Case H-18 (fig. 5). Born co-twin to normal bull. Killed October 14, 1913, aged thirty-one days. External genitalia typically female. Vagina and uterus quite rudimentary. Seminal vesicles and parts of Wolffian ducts (solid cords) present. Data incomplete on position of the gonads, but they were probably in the ovarian position. No spermatic cord.

Case H-42. Born co-twin with a normal bull. Aged about eighteen months. External genitalia typically female. Vagina



and uterus quite rudimentary. Vas deferens (lower half) and seminal vesicles present. Gonads rudimentary and lying in ovarian position. No spermatic cord. Owing to the atypical organization of the sex-cord region in this case, it will be considered separately at the end of the discussion of the other three cases.

*The general topography of the gonads.* These four gonads are very small, oval structures (fig. 5). For comparative sizes and other data, consult tables 1, 2, and 3. They are in reality rudimentary testes, which have failed to descend, and thus retain their embryonic position, i.e., the position normal for ovaries. Like the gonads having a low degree of transformation in the male direction, these possess the majority of the parts typical of a testis. Entering the anterior end and projecting nearly to the posterior end of the reproductive gland in each case, is the rete. It forms an eccentrically placed core, about which are the sexual cords and connective-tissue stroma containing interstitial cells. Surrounding the entire gonad is a capsule of connective-tissue fibers, the tunica albuginea. The anterior end may be further modified by the presence of a well-developed vascular plexus (cases 42 and 66) and of a few very definite epididymal tubules (cases 42 and H-42).

*The tunica albuginea and tunica vasculosa.* Without exception, the free-martin gonad is enclosed by a capsule of connective-tissue fibers, but the relative thickness of the capsule and the compactness of its fibers vary in the different groups of cases. In the specimens under consideration, the fibers are coarse, compactly arranged in the outer layer, and run parallel with each other as they encircle the gonad (fig. 5). If these fibers are compared with corresponding fibers in the tunica albuginea of a mature foetal testis, the latter are seen to be more closely packed together and the individual fibers finer. The capsule of the mature foetal testis is thirteen times thicker than that of the free-martin gonads of this group.

In some of the free-martin gonads of this group, the inner portion of the connective-tissue capsule is characterized by a loose arrangement of the fibers. In cases 66 and 42 a few blood-vessels make their appearance in this layer, forming a primor-



dium of the tunica vasculosa. This primordium shows a degree of differentiation comparable to the condition of the tunica vasculosa of a normal testis of a 4.8-cm. embryo. Blood-vessels from this vascular zone pass to the stroma of the sex-cord region, supplying it with blood. In case H-18 no tunica vasculosa is apparent, so that the blood-vessels enter the hilus and are distributed in a centrifugal manner to the rete tubules and the sex-cord region (fig. 5).

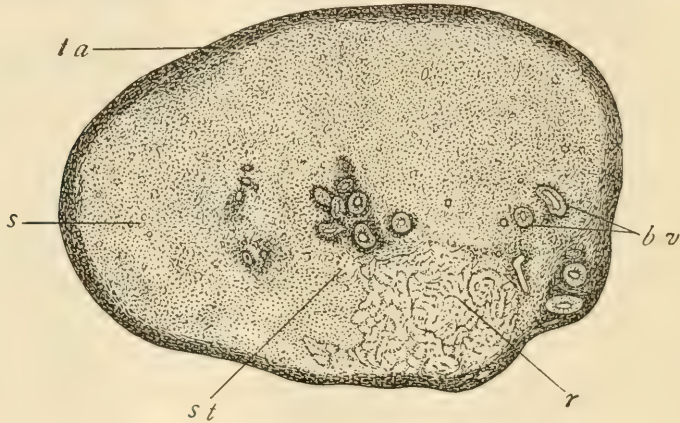


Fig. 5 Transverse section through free-martin gonad, H-18. *bv*, blood-vessels; *r*, rete (note its eccentric position); *s*, seminiferous tubule; *st*, tubuli recti connecting rete tubules and seminiferous tubules; *ta*, tunica albuginea (note absence of tunica vasculosa).  $\times 14$ .

These two ways for the distribution of the blood in the free-martin gonads will be understood from the normal arrangement of the blood-vessels in the ovary and in the testis, and in the light of the fact that the free-martin gonad is primarily an ovary which is being transformed into a testis. A comparative study of the normal method of distribution in ovaries and testes shows a marked difference. In the testis blood-vessels enter the anterior end, ramify extensively throughout the tunica vasculosa, from which they send branches inwardly, in a centripetal manner, to the seminiferous tubules and rete tubules (fig. 4). In the ovary the blood-vessels are distributed in a centrifugal

manner; the blood-vessels enter the hilus (fig. 3), from which they are distributed outwardly to the medullary cords and cortex.

It is thus seen that the distribution of the blood in a centripetal manner is characteristically a male feature, while the centrifugal method is strictly a female attribute. Since the free-martin gonad is an ovary which is changing into a testis, as has been shown, it is not an unexpected finding that the manner of the distribution of the blood-vessels should also change in the male direction. This is exactly what is found in the free-martin gonads of cases 66 and 42, where the blood is distributed in a centripetal manner to the sex-cord region. On the other hand, in case H-18 the original ovarian method is retained, so that the blood is distributed in a centrifugal manner to the sex-cord region.

The peritoneal surface of the gonad consists of a single layer of cuboidal epithelial cells in case 66, and of a single layer of flattened epithelial cells in cases 42, H-42, and H-18. Instead of the thick germinal epithelium of the normal ovary, these, as well as all other free-martin gonads, are enclosed within a single layer of more or less flattened epithelial cells as in the normal testis.

*The sex-cord region.* In this group of gonads (except in H-42, which is atypical and will be considered separately) the sex-cord region constitutes the largest portion of the gonad and shows a moderate degree of organization of the sexual cords and intercordal tissue. The latter comprises a stroma of a moderate number of loosely arranged connective-tissue fibers, and between them the so-called interstitial cells. The fibers of the stroma are continuous with the connective-tissue fibers of the tunica albuginea and ramify throughout the sex-cord region, forming a framework between the sexual cords. Histologically, the stroma is composed of fusiform-shaped cells, which are apparently fibroblasts of connective tissue. These cells are further characterized by elongated and very prominent nuclei, but the cytoplasm is scanty and quite inconspicuous. From the ends of the nuclei extend fibrillar processes, which anastomose,

with similar processes attached to other nuclei to form the network of the stroma. In this loose network are the interstitial cells, which, in the different gonads of this group vary greatly as to size, shape, and structure. In gonad H-18 they are relatively few in number, and possess large, round nuclei and a homogeneous cytoplasm containing a few fuchsinophilic granules. In gonads 42 and 66 the interstitial cells are difficult to recognize, owing to the presence of large masses of cells which at ruptured places in the walls of the sexual cords are continuous with the supporting epithelial cells of the sexual cords. The cells within the cords are identical in shape and structure with the cells of these masses. It is not at all improbable that some of these masses of cells lose their specificity as supporting epithelial cells and become interstitial cells as a similar process occurs in degenerating follicles of the ovary of a normal mature foetus, where the granulosa cells transform into masses of cells which resemble interstitial cells.

Two clearly different degrees in the organization of the supporting epithelial cells may be recognized. They are either arranged in definite cords or they occur in unorganized masses of cells. The latter are particularly abundant and distinct in the gonad of case 66, less abundant in case 42, and least in H-18. The close resemblance of these cells to the supporting epithelial cells within the sexual cords and their close association with the sexual cords suggest their origin from the latter. Similar masses of unorganized supporting epithelial cells, in addition to the medullary cords, occur in the normal ovary (N 15, 18 cm.). Whether or not these unorganized masses in the free-martin gonads transform into sexual cords cannot be determined from the data at hand, but it is suggested that they do, since these masses are most numerous and largest in the youngest gonad (case 66). Obviously, there is a tendency for these unorganized masses to disappear with increasing age. However, differentiation is not entirely the result of aging, but is dependent also on the variability in the action of the sex hormones. For example, in the gonads of the oldest free-martin examined, the differentiation of the sex-cord region is much less than in these younger gonads.



The sexual cords are of two types, those resembling medullary cords and those resembling seminiferous tubules. The former resemble the medullary cords of the normal ovary in that they are solid strands (cords) of irregularly arranged and closely packed supporting epithelial cells forming a syncytium, which may be regarded as the primordium of a Sertoli-cell syncytium. In the free-martin, germ cells are absent from the medullary cords. These sexual cords are not only structurally different, but they are smaller than the sexual cords which resemble seminiferous tubules. Cases 66 and 42 show distinct transitional stages in the transformation of medullary cords into seminiferous tubules through increase in size and rearrangement of the nuclei of the syncytium.

In such transitional stages, the nuclei are two and sometimes three layers deep, and less crowded together than in the medullary cords and accompanied by a slight increase in the size of the cords. In the most completely transformed sexual cords (seminiferous tubules), there is a further increase in size accompanied by the arrangement of the nuclei of the supporting epithelium into a single layer at the periphery. The oval nuclei of this layer are arranged with their long axes perpendicular to the wall of the tube. Extending from the inner ends of the nuclei are strands of cytoplasm, all of which meet at the center of the seminiferous tubule (fig. 6, *s*). Such a center has a potentiality of forming a lumen, as in the normal seminiferous tubule (figs. 7 and 8). If this syncytium of supporting epithelial cells is compared with that of the mature foetal testis (fig. 7) and a young calf testis (fig. 8), a close structural similarity will be seen, with one important deficiency, namely, that germ cells of any stage are absent from the syncytium of the free-martin seminiferous tubule. It may also be noted that in the tubules of the free-martin there is much less regularity in the arrangement of the nuclei of the supporting epithelial cells; in this respect the arrangement is like that of very young foetal testes (table 3).

Each wall of these sexual cords is composed of a basement membrane upon which the supporting epithelial cells rest, and external



to which is a variable number of concentrically arranged lamellae of connective-tissue fibers. As a rule, the number of lamellae increases as the medullary type of cord is transformed into that of a seminiferous tubule type. The wall of a normal seminiferous tubule has a similar composition, with many lamellae.

In order to understand the changes undergone by a medullary cord of the free-martin gonad as it transforms into a seminiferous tubule, it will be necessary to consider briefly the structure



Fig. 6 Portion of a transverse section through the sex-cord region of the free-martin gonad, 66. *bv*, blood-vessel containing corpuscles; *i*, interstitial cells; *s*, sexual cord which resembles a seminiferous tubule (note arrangement of the nuclei of the supporting epithelial syncytium, and strands of cytoplasm extending centrally from the inner ends of the nuclei); *ts*, sexual cord which shows a transitional stage between a medullary cord and a seminiferous tubule (note arrangement of nuclei within syncytium); *ct*, connective tissue.  $\times 380$ .

of the medullary cords of the normal embryonic ovary, and also to follow the development of the syncytium of supporting epithelial cells in normal embryonic testes and in three post-natal testes.

Examination of an ovary of a normal 8.3-cm. embryo (N 7), shows that the medullary cords consist of solid strands of supporting epithelial cells enclosed within definite walls. The cells are closely crowded and irregularly arranged, and owing to the absence of cell walls they form a syncytium. In a medullary cord of an ovary from an 18-cm. embryo there is some

rearrangement of these cells, as the comparatively large nuclei tend to be crowded into a single layer at the periphery. This leaves an area of the syncytium more or less free of nuclei in the center of the cord, but no lumen is formed. Examination of older embryonic ovaries shows similar conditions. This is the

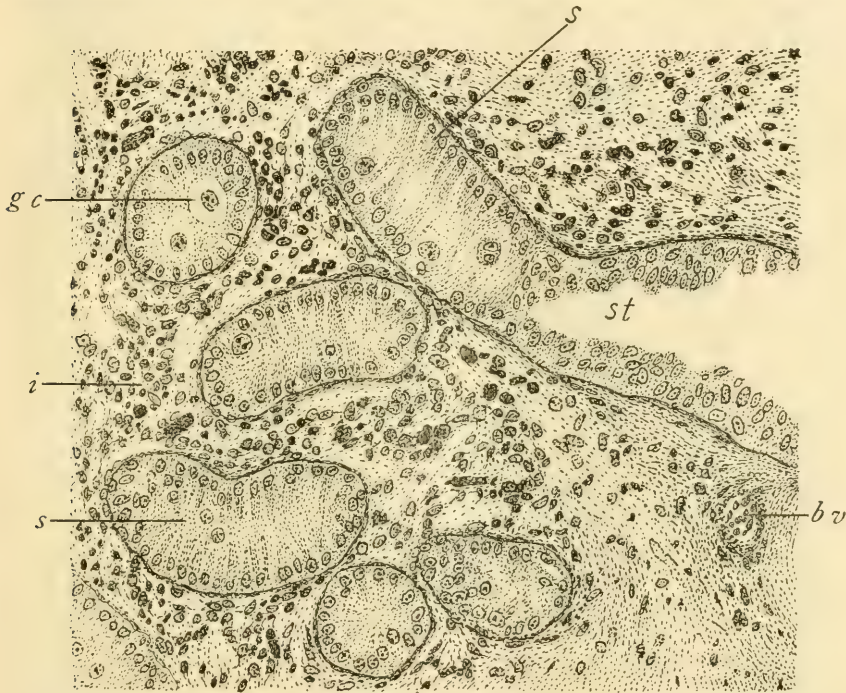


Fig. 7 Portion of a transverse section through the testis of a mature male fetus (89 cm.), Bos 3. *bv*, blood-vessel; *gc*, primordial germ cell; *s*, seminiferous tubule showing nuclei of the supporting epithelial syncytium arranged into a single layer at the periphery; strands of cytoplasm extending from the inner ends of the oval nuclei toward the center of the tubule; *st*, tubulus rectus (straight tubule) in conjunction with a seminiferous tubule; *i*, inter-tubular tissue.  $\times 307$ .

normal limit of differentiation, as they are destined to degenerate; no medullary cords are evident in ovaries from birth on.

In the youngest normal testis examined (4.8 cm., N 13) the sexual cords are unorganized, as the walls can only be distinguished with difficulty and the supporting epithelial cells are

irregularly arranged. In testes from a 7-cm. (N 10) and a 12.7-cm. (T 16) embryo some slight degree of organization is recognizable. A definite tubule wall is evident, and the supporting epithelial cells are formed in a syncytium, the nuclei of which are becoming arranged into layers, some having three layers, others two layers. From the inner ends of the nuclei, which

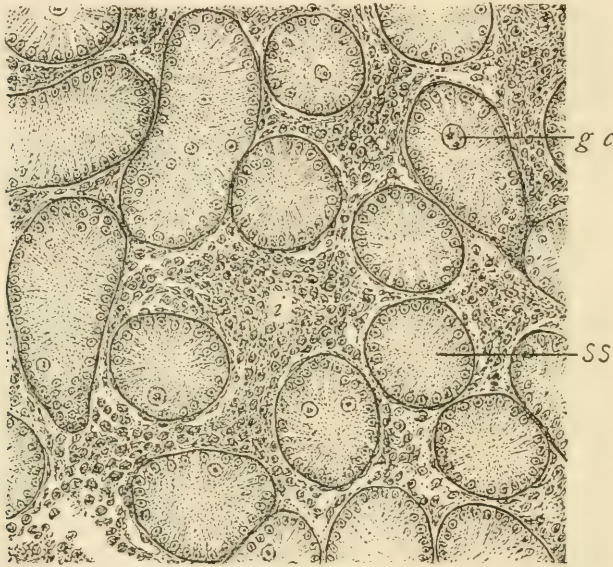


Fig. 8 Portion of a transverse section through a testis of a young male calf (six months?), Bos 12. *gc*, primordial germ cell; *ss*, syncytium of supporting epithelial cells; *i*, interstitial cells.  $\times 307$ .

show a tendency to be arranged with their long axes perpendicular to the tubule wall, strands of cytoplasm extend toward the center of the tubule. The organization of the syncytium progresses in the older testes, until finally a striking degree of regularity is shown in testes from 31-cm., 61-cm., 89-cm. embryos and in testes from young calves (2 and 12 days, and 6 months). Figures 7 and 8 show the nuclei of the supporting epithelial syncytium arranged in a single distinct layer, closely applied to the wall of the tubule. The long axes of the oval nuclei are



parallel to each other and perpendicular to the tube wall. From the inner end of each nucleus extends a strand of cytoplasm toward the center of the tubule; the point where these strands center has the potentiality of forming a lumen. Within this syncytium are found a few large, clear cells—the primordial germ cells.

Bearing in mind the history of the medullary cords and the normal development of the seminiferous tubules, the development of the seminiferous tubule in the free-martin is comprehensible. The medullary type of sexual cord resembles not only the medullary cords of the normal ovary, but also the youngest sexual cords in the testis, these being homologous structures. As a sexual cord in the free-martin, originally of medullary type, transforms into a seminiferous tubule, it passes through stages which are in essentials similar to those in the normal development of the seminiferous tubule.

The sex-cord region of H-42. The sex-cord region of this gonad presents a striking contrast to the sex-cord region of other gonads of the entire series, in showing a few female elements. This region is smaller and much more poorly organized than in the other cases of this group of moderately transformed gonads. It is made up chiefly of masses of interstitial tissue (connective-tissue stroma and interstitial cells), in which are scattered sexual cords. Of the two types of sexual cords, the larger number here resemble seminiferous tubules, while a few are distinctly female, possessing primary follicles containing germ cells. These sexual cords which resemble seminiferous tubules show different degrees of organization of the syncytium of supporting epithelial cells; in some the nuclei are arranged in a single layer and in others they are irregularly arranged and closely packed. The walls of these sexual cords are thickened, otherwise they agree very closely in structure with the sexual cords of cases 66, 42, and H-18 described above, and therefore will not be considered further.

The sexual cords which appear to be female in structure are located largely near the periphery of the sex-cord region. They are composed of a typical egg cell surrounded by a single layer of more or less flattened follicular epithelial cells. In some more



than one egg is present. Such primary follicles are enclosed by a thickened wall of connective-tissue fibers, which is not unlike the wall of the male sexual cords. Magnusson ('18) described large vesicle-like structures which in six different cases appeared to resemble Graafian follicles, but in none did he find germ cells.

The interstitial cells are of two types, those which resemble the interstitial cells of the normal testis and of other free-martin gonads and those which resemble lutein cells of the corpora lutea in cattle. The latter are of large size, and the cytoplasm is filled with large granules staining yellow with Mallory's 'triple stain.' These lutein-like cells usually occur in localized masses distributed chiefly at the periphery of the sex-cord region. One of these masses is a large, rounded collection enclosed by a connective-tissue capsule, which measures 1 mm. in diameter, and resembles closely a miniature corpus luteum. Magnusson ('18) described three gonads in which similar bodies of lutein-like cells were found. He found even larger bodies, as he states on page 47, " . . . einen verhältnismässig grossen intensiv gelben Körper von 3 mm. Durchmesser ein, der von einer fibrösen Kapsel umgeben war." The origin of these lutein-like cells cannot be decided from the meager evidence furnished by this one case, but it is not improbable that they arise from the follicular (granulosa) cells of atretic follicles.

The occurrence of primary follicles in the sex-cord region raises the question of their origin. There are two possible explanations of their occurrence. In the first place, a few of the cords of Pflüger may have invaginated before the optimum conditions for the action of the male sex hormones were established. This view is supported by the peripheral position of the majority of the primary follicles and masses of lutein cells, which probably arise from the follicles. Secondly, such follicles may have arisen from the 'medullary follicles' of the ovary. The evidence is good for this point of view, since the sex-cord region is comparatively small, and it ought to be larger if any of the cords of Pflüger had invaginated. Allen ('04) and others have described simple follicles containing germ cells in the medullary cords of foetal ovaries. Such follicles with germ cells are also present in

the medullary cords of foetal ovaries in cattle. It may be that the germ cells which are present in the sexual cords of the foetal free-martin gonads persist to form follicles, instead of degenerating as in all of the other free-martin gonads examined. (For a further consideration of the fate of these germ cells, the general discussion may be consulted).

*The rete tubules and connections.* The rete region in each free-martin gonad of this group is a distinct cord of tubules, which enters the anterior end and penetrates nearly to the posterior end of the gonad. It retains its primitive relation to the sex-cord region by its eccentric position. In other words, although the sex-cord region is large and moderately well organized, it has not grown sufficiently to entirely surround the rete, so that the rete lies next to the tunica albuginea (fig. 5) as in the gonads exhibiting a low degree of transformation. The structure of the rete tubules resembles the condition described previously for gonads having a low degree of transformation.

The relationships of the rete tubules to the sexual cords and to the tubules of the epididymis are important indices of the degree of transformation in the male direction. In the normal ovary no branches of the rete establish connections with the medullary cords (fig. 3) but in the normal testis connections between the seminiferous tubules are formed by lateral branches (tubuli recti) of the rete tubules extending to meet the seminiferous tubules (figs. 4 and 7, *st*). If such branches of the rete tubules make connections with the sexual cords in the free-martin gonad, it is a further indication of the assumption of male characters. This is exactly what is found in this group of free-martin gonads, although the number of tubuli recti is comparatively small. Such connections are shown in figure 5, *st*.

The relation of the rete tubules to the tubules of the epididymis is clearly shown in case 42 (fig. 9, *rt, e*). This figure illustrates a transverse section just anterior to the sex-cord region, and shows rete tubules extending to establish connections with a tubule of the vasa efferentia. There are four of these tubules; they are short, closed at both ends, and lined with ciliated epithelial cells, which rest upon a basement membrane enclosed

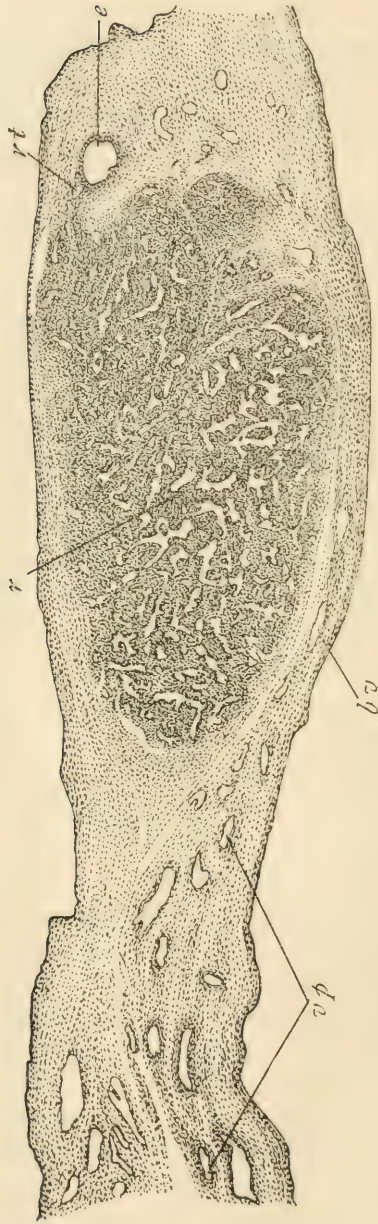


Fig. 9 Transverse section through anterior end of free-martin gonad, 42. *bv*, blood vessel; *e*, epididymal tubule (vas efferens); *rt*, rete tubules; *rt*, rete tubules connecting with epididymal tubule; *v*, vas efferens; *vp*, vascular plexus.  $\times 32$ .



by a layer of circular smooth muscle fibers. Similar connections are shown in case H-42, but the number of vasa efferentia tubules is larger (about twenty in transverse section). In neither case can traces of a Wolffian duct connecting with the vasa efferentia be found. These conditions will be understood from a comparison with the normal relationships of these parts in the ovary and testis.

Although the rete tubules at the anterior end of a normal foetal ovary (6.3 cm., N 14) may establish connections with the renal corpuscles of the Wolffian body, these connections are not of much significance, owing to the early degeneration of the Wolffian body in the female. However, in the male these connections are permanent, owing to their persistence as the vasa efferentia of the epididymis. The vasa efferentia are connected with the upper portion of the vas deferens (Wolffian duct), which is known as the ductus epididymis. The vasa efferentia and the ductus epididymis are both much contorted tubules and constitute the head (*globus major*) of the epididymis, which fits over the anterior end of the testis. From the head of the epididymis the coiled vas deferens passes posteriorly as the body of the epididymis (*corpus epididymidis*) to the posterior end of the testis where it forms a conical mass, the tail (*globus minor*) of the epididymis. From the tail of the epididymis, the vas deferens passes anteriorly along the side of the testis to the spermatic cord.

From the above accounts, it will be seen that in the free-martin gonads of this group (42 and H-42) the rete tubules establish connections with the vasa efferentia tubules of the epididymis, and constitute the beginnings of the head of an epididymis, but as the vas deferens is absent, this remains rudimentary.

*The spermatic cord.* In gonads 42 and 66 a rudiment of a spermatic cord occurs. Broadly attached at the hilus near the anterior end, and at the side of the rete opposite the epididymis, is a flattened cord of blood-vessels—the vascular cord (fig. 9, *vp*). Structurally, it is a plexus of blood-vessels embedded in connective-tissue fibers. The fibromuscular wall of the blood-vessels

includes both circular and longitudinal fibers. In relative position and structure it resembles the vascular cord of the normal spermatic cord. The vascular cord, vas deferens, nerves, and lymphatics are bound together by connective tissue to form the spermatic cord of the normal male. Obviously, in these free-martin gonads (cases 42 and 66), the spermatic cord is rudimentary, owing to the presence of only one constituent part—the vascular cord.

*C. Gonads exhibiting a high degree of transformation*

This group illustrates the highest degree of transformation in the male direction of all the free-martin gonads examined. That they are well-developed male sex glands is shown by the following characters: the majority of the gonads are descended to a position in the groin; the seminiferous tubules are well differentiated; tubuli recti (straight tubules) connect the rete tubules with the seminiferous tubules; the rete tubules connect with the vasa efferentia; the epididymis is typically male, and a typical spermatic cord is present.

Histories of the three cases to be examined:

Case 44. Born co-twin with a normal male March 12, 1916, of Holstein-Friesian parentage. Slaughtered April 29, 1916. Gonads descended into peritoneal sacs lying between the skin and the abdominal muscles in the region of the groin. No trace of vagina, uterus or tubes. No scrotum. External genitalia typically female. Vasa deferentia open into dorsal wall of urinogenital sinus, from thence to the gonads. Seminal vesicles lateral to bases of vas deferens. Spermatic cord typically male.

Case H-46 (fig. 10). Born co-twin with a normal bull. Slaughtered when five and one-half weeks old. Gonads in ovarian position. Vagina rudimentary, bases of Müllerian ducts fused (uterus), free horns of uterus short. Vasa deferentia extend from rudimentary vagina nearly to gonads—present again along gonad. No seminal vesicles. External genitalia typically female. Distinct rounded vascular cord.

Case H-37. Co-twin of normal bull. Born October, 1915, slaughtered December, 1916. Large gonads descended through

abdominal wall to a position immediately under the skin in the region of the groin. No udder development, but the teats were large. No scrotum. External genitalia female except the clitoris, which is transformed into a penis-like structure posteriorly directed. Vas deferens led from epididymis, which is normally related to the gonad, to urethra. Seminal vesicles present, but only the left has a duct which lacks a lumen. Spermatic cord normal in appearance. Rudimentary prostate.

*The general topography of the gonads.* These three highly transformed gonads are comparatively larger than gonads less transformed, but smaller than normal testes of the same age. The gonad of H-37, the most completely transformed of the entire series, is approximately the same size as a testis of a six-months-old calf (40 x 12 mm.). The testis of a bull of about the same age as this free-martin measures 125 x 57 mm. In cases 44 and H-37, the left gonad is much larger than the right gonad (table 1 for details). Not only has the size increased, but the shape has changed from ovoid to oblong.

In contrast to the other groups, the ovarian position of these gonads is not retained (except H-46), but they have descended into peritoneal sacs, which instead of entering a scrotum, as the normal testes do, are retained between the abdominal muscles and the skin in the region of the groin. Even this partial descent is a distinct indication of a further transformation in the male direction of these free-martin gonads.

The general morphological relationships of the parts are like that of a normal testis. Each gonad is enclosed by a thickened capsule, the tunica albuginea. The rete enters the anterior end, extends toward and in two cases reaches the posterior end of the sex-cord region. It forms an eccentrically placed core about which are found the seminiferous tubules and intertubular tissue. Many tubuli recti connect the rete tubules with the seminiferous tubules. At the anterior end of the gonad the rete tubules establish connections with a well-developed head of the epididymis. The body and tail of the epididymis bear the typical male relationships to the gonad. In each case there is a well-developed vascular cord which is attached to the anterior



end of the gonad; this vascular cord is loosely bound with a vas deferens to form a spermatic cord.

*The tunica albuginea and tunica vasculosa.* A study of the connective tissue capsule of these three gonads indicates that it also may be rather completely transformed in the male direction. The average thickness of the capsule is about 0.8 mm. which is several times thicker than in the capsules from gonads less transformed, and which approaches very closely the normal thickness of 1 mm. in the testis of an adult. As in the other groups, the inner layer of the connective-tissue capsule is modified by the addition of blood-vessels, constituting therefore a vascular zone. This zone varies in the different gonads of this group. In case 44 it is poorly formed, yet at places a distinct layer is present containing blood-vessels which pass in a centripetal direction to the sexual cords. In addition, blood-vessels also enter the hilus to be distributed in a centrifugal manner to the sexual cords. In case H-46 (fig. 10, *tv*) is seen a very definite vascular zone, from which blood-vessels are distributed centripetally to the sex-cord region. The existence of the tunica vasculosa in case H-37 as a distinct layer is lost by its close mergence with the outer layer, the two constituting the tunica albuginea, the inner portion of which contains the blood-vessels. In contrast to the coarse condition of the fibers in less transformed gonads, the fibers are fine in H-37. The fineness of the fibers and the mergence of the two layers into one tunica albuginea, resemble in close detail the structure of the normal testis (table 3).

Upon the tunica albuginea is a single layer of flattened epithelial cells, which constitutes the visceral layer of the tunica vaginalis.

*The sex-cord region.* The sex-cord regions of these three free-martin gonads have reached the highest degree of differentiation, organization, and largest size of any of the post-natal gonads. The primitive relationships of the sex-cord region to the core of rete tubules is shown by the eccentric position of the latter. The sex-cord region completely surrounds the rete cord, and this is a distinct advance in the male direction where the

rete normally forms a core in the center of the testis (fig. 4). In no case has the sex-cord region advanced to this condition, but a definite step in that direction is seen in case H-37, and toward the posterior end of gonad H-46 where the sex-cord

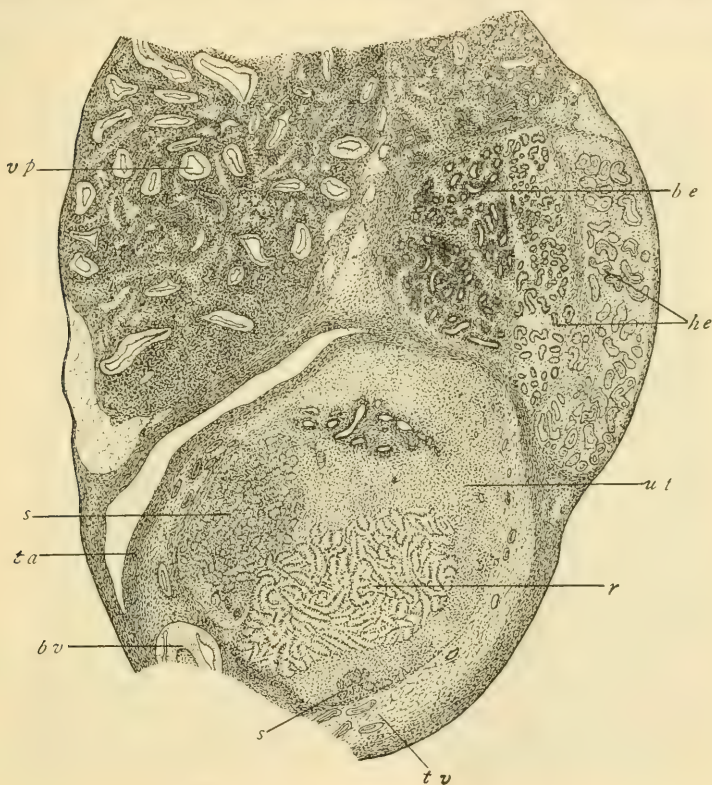


Fig. 10 Transverse section through gonad, vascular plexus and epididymis of free-martin, H-46. *bv*, blood-vessel; *be*, tubules of the body of the epididymis; *he*, tubules of the head of the epididymis; *r*, rete tubules; *s*, seminiferous tubules; *ta*, tunica albuginea; *tv*, tunica vasculosa; *ut*, 'undifferentiated tissue'; *vp*, plexus of blood-vessels.  $\times 11$ .

region has grown around the rete to a degree such that the rete no longer touches the tunica albuginea, but has shifted toward the center of the gonad. Nevertheless, it is still eccentrically situated with respect to the greater portion of the sex-cord

region. In the other two cases of this group, the sex-cord region, although well developed, does not lie between the rete and the connective-tissue capsule. In other words, the rete still lies in its primitive position at the hilus (fig. 10, *r*).

As in the other free-martin gonads, the intertubular tissue is composed of a stroma of connective-tissue fibers among which



Fig. 11 Portion of the sex-cord region of the free-martin gonad, H-37. *bv*, blood vessel; *ss*, Sertoli-cell syncytium (no germ cells) of a seminiferous tubule; *i*, interstitial cells; *w*, thickened wall of tubule.  $\times 107$ .

are interstitial cells. A noticeable difference, and one probably of much significance, is the great abundance of these interstitial cells. The interstitial cells are most abundant in the highest transformed gonads. In case H-37 (fig. 11, *i*), which is the most completely transformed gonad of all of the post-natal free-martin gonads, these cells are exceedingly abundant and of large size.



Particularly large collections are found around blood-vessels and at the 'carrefours,' that is, in the intervals between the sections of three or more seminiferous tubules. These large areas may be observed with the unaided eye. They are not isolated collections, but are continuous with the interstitial cells in the stroma strands between the tubules. In cases 44 and H-46 (fig. 12, *i*) the interstitial cells are moderately numerous.

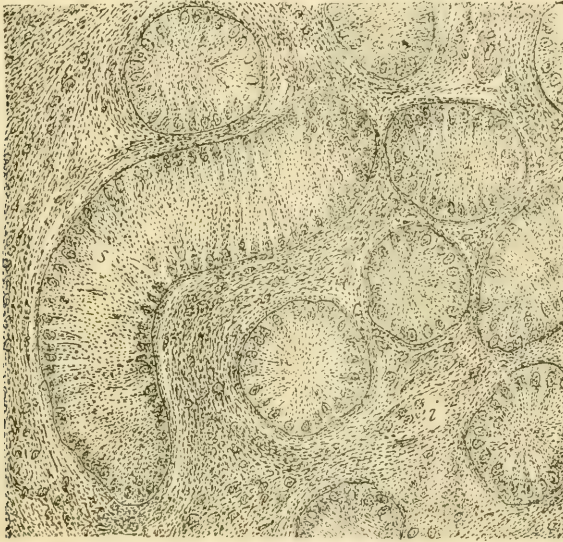


Fig. 12 Portion of the sex-cord region of free-martin gonad, H-46. *i*, interstitial cells in a connective-tissue stroma; *s*, seminiferous tubule showing the nuclei of the supporting epithelial syncytium arranged into a single layer closely applied to the wall of the tubule. From the inner end of each nucleus extends a strand of cytoplasm toward the center of the tubule. Note absence of germ cells.  $\times 307$ .

A detailed examination of the interstitial cells in case H-37 (fig. 11, *i*) shows much variation in their structural features. There is much variation in size, the majority being many times larger than the interstitial cells of the normal testis of the adult. In shape, the majority are irregularly polygonal. The eccentrically placed nucleus is large, somewhat vesicular, and contains scattered chromatic granules. The structure of the cytoplasm also varies in the different types of cells. In a small proportion

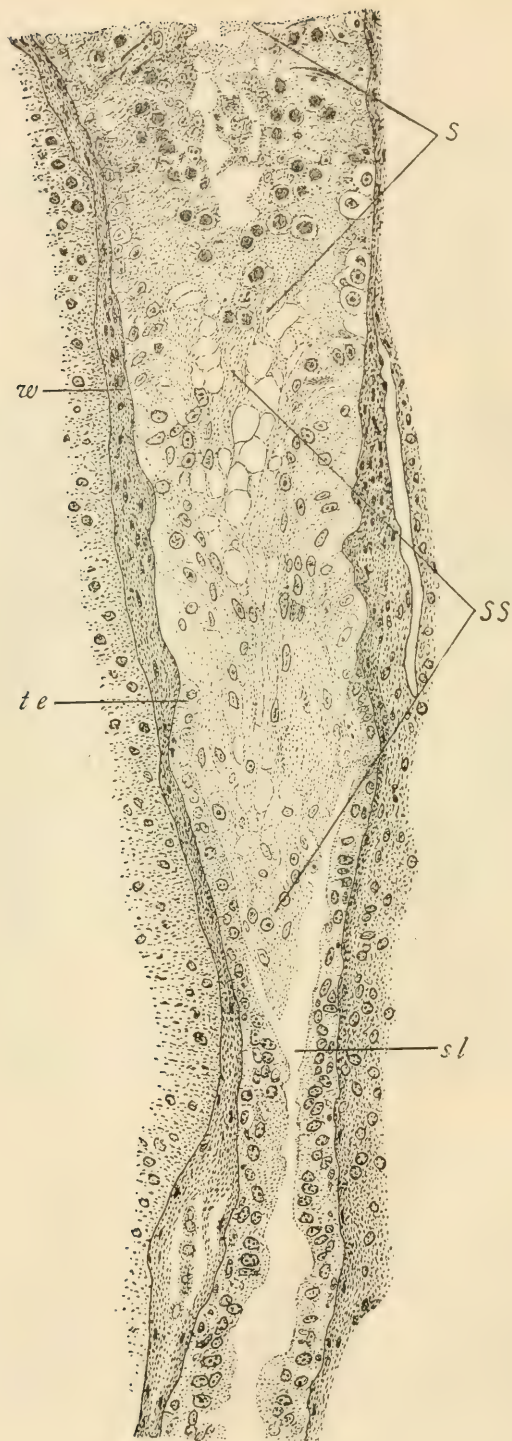


Figure 13

it is homogeneous throughout, in a few others it is extensively vacuolated, while in the majority of the cells the cytoplasm contains bundles of rod-like structures. Owing to the poor fixation of the gonads from cases 44 and H-46, a detailed description of the interstitial cells cannot be given.

The organization of the sexual cords has reached its highest degree of transformation in the male direction, so that a majority of the sexual cords are definite convoluted seminiferous tubules. In H-37 the tubules are scattered more or less evenly throughout the sex-cord region, but in H-46 and 44 there are areas which are free from tubules. They appear as areas of apparently undifferentiated tissue (fig. 10, *ut*), but on closer examination it is seen that some organization has occurred. Among the abundant connective-tissue fibers are slender cords of sexual cords, a few of which resemble miniature seminiferous tubules and others which resemble medullary cords, and interstitial cells. Owing to the close resemblance of these undifferentiated areas to the sex-cord region of some of the foetal free-martin gonads, they are regarded as areas which retain the embryonic structure.

The seminiferous tubules appear to be as much coiled as the normal tubules of the testis, but they are not as closely packed together nor as large. The tubules of H-37 (fig. 11), which resemble to a close degree the tubules of the normal testis of the adult, are, however, only half as large in diameter as the latter (adult 0.225 mm.) (fig. 13, *s*). The tubules of cases 44 and H-46 (fig. 12) are about one-fourth the diameter of a normal tubule of the adult testis.

Each tubule is enclosed by a much thickened membranous wall, which is composed of a number of concentric lamellae of connective-tissue fibers. The innermost lamellae are very closely arranged and hyaline-like, giving the appearance of a

Fig. 13 The junction of a seminiferous tubule and a straight tubule (*tubulus rectus*) from the testis of an adult bull, Bos 2. *s*, seminiferous tubule showing spermatogonia, maturing germ cells and spermatozoa; *sl*, lumen of straight tubule; *ss*, Sertoli-cell syncytium free from germ cells; *te*, abrupt beginning of the epithelium of the straight tubule; *w*, connective-tissue wall of the tubule.  $\times 240$ .



much thickened basement membrane. The outer lamellae are loosely arranged and blend with the connective-tissue fibers of the stroma.

Within the membranous wall the supporting epithelium is arranged in such a manner that two distinct stages in organization may be recognized. The first stage is illustrated in the gonads from cases 44 and H-46 (fig. 12, *s*); here the nuclei of the supporting epithelial syncytium are very regularly arranged into a single layer. The long axes of the oval nuclei are perpendicular to the tubular wall, and from the inner ends of the nuclei filmy strands of cytoplasm extend toward the center of the tubule. This syncytium of epithelial cells is identical with the syncytium found in the seminiferous tubules of late foetal testes and young postnatal testes (compare with figures 7 and 8), with one important exception, namely, the complete absence of germ cells in the free-martin syncytium.

The second stage, illustrated by case H-37 (fig. 11, *ss*), shows more advanced differentiation in the male direction, as the supporting epithelial syncytium has become resolved into a Sertoli-cell syncytium as in the adult normal testis (compare with fig. 13, *ss*). The cytoplasmic substance of the syncytium stains lightly, and structurally it appears as a loose meshwork of branching strands of cytoplasm. These strands have more or less of a fibrillar structure, the fibrils of which are very delicate and in some cases not very sharply defined. The cytoplasm is continuous throughout the tubules except as interrupted by the scattered nuclei and spaces. The oval nuclei, which lie at various levels in the syncytium, are characterized by their distinctive nucleoli. The nucleolus is a comparatively large, nearly spherical body which takes a deep chromatic stain. Apart from the nucleolus, the chromatic material is very scanty. The Sertoli-cell syncytium is structurally alike in both the free-martin seminiferous tubule and the normal seminiferous tubule of the adult bull, except male sex cells are absent in the former. The structure of the normal syncytium can be very beautifully demonstrated at the zone of junction between a seminiferous tubule and a straight tubule (fig. 13, *ss*). In this zone the

Sertoli-cell syncytium is free from male germ cells of all stages, so that its normal structure can be easily observed. This is a point of considerable interest, as it is the only region in the seminiferous tubule that furnishes an unobstructed view of the normal syncytium. So far as the writer is aware, this zone has not been used before in describing the normal structure of the syncytium.

*The rete tubules and tubuli recti.* As in the other free-martin gonads, the rete region is an eccentrically placed core of anastomosing tubules, which enters the anterior end of the gonad and projects posteriorly. The distance that it penetrates posteriorly into the sex-cord region varies; in case 44 it penetrates about half the length of the gonad, in case H-46 it penetrates to the posterior end of the sex-cord region, probably also in case H-37 (the extent was not determined, but owing to the extreme transformation of the gonad, it is probable that it penetrated to the posterior end). The eccentric position of the rete has been considered above under the description of the sex-cord region.

The rete of the free-martin gonad, like that of the normal testis, consists of a network of strands of coarse connective-tissue fibers, in which are anastomosing tubules. The strands of connective tissue are as wide in H-37 as in the normal rete testis, but in cases 44 and H-46 are comparatively narrow. Resting upon the wall of the rete tubule is a lining of columnar epithelial cells, which resemble in a very striking degree the normal condition (compare figures 14 and 15). The lumina of the tubules are wider in more highly transformed gonads, so that the rete region appears less compact than in the less transformed gonads.

In each gonad of this group branches of the rete tubules have established connections with the seminiferous tubules. Figure 16 shows the abrupt transition from the seminiferous tubule into a straight tubule in H-37. The seminiferous tubule is filled with a stringy syncytium of Sertoli cells, while the straight tubule contains a lumen and is lined with low columnar epithelial cells. Comparison with figure 13 shows the transitional zones to be similar. These straight tubules are most numerous in H-37,

the gonad which is most transformed in the male direction. In cases H-46 and 44 they are less numerous than in H-37, but more numerous than in less transformed gonads. Obviously, there exists a graded series in the development of the straight



Fig. 14 Rete tubules from testis of a young male calf (six months?), Bos 12. *l*, lumen of tubule; *re*, epithelium of tubule; *rs*, connective tissue stroma between rete tubules.  $\times 307$ .

tubules, the moderately transformed gonads having a few, and the most completely transformed gonads having many.

*The epididymis.* Each of the three gonads of this group possesses a well-developed epididymis, which marks a pronounced step in the direction toward male organization of the free-martin gonad. Although the epididymis bears a relation to the reproductive gland, which is approximately normal, its



parts are misshaped and enlarged. Only a small portion of the epididymis of H-37 was examined, but Doctor Cole in a communication states that it "bore the normal relation to each testicle." In each of the gonads examined (H-46 and 44) the rete continues anteriorly a short distance beyond the sex-cord

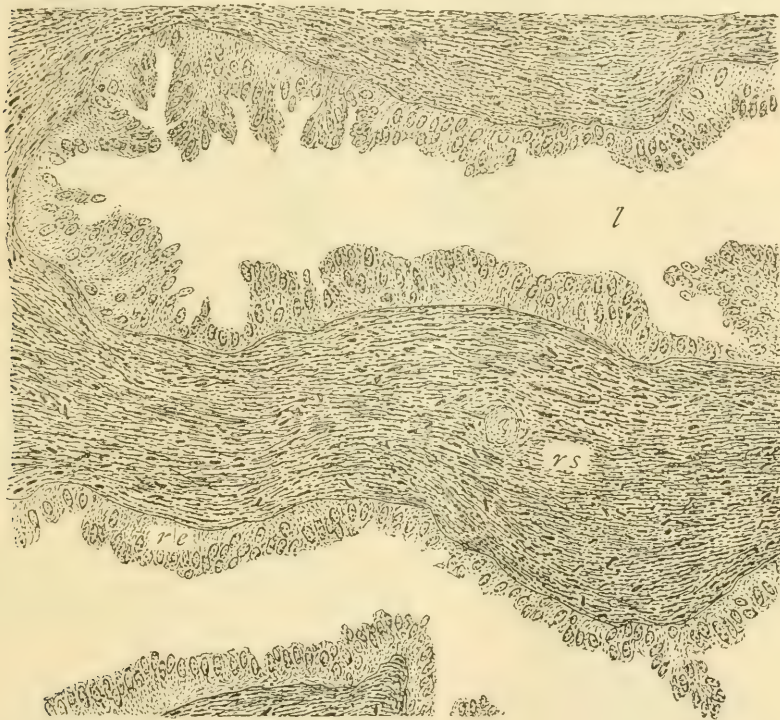


Fig. 15 Rete tubules from gonad of free-martin, H-37. *l*, lumen of tubule; *re*, epithelium of tubule; *rs*, connective tissue stroma of the rete.  $\times 307$ .

region to make connections with an elongated lobe of coiled epididymal tubules. This elongated lobe constitutes the head (globus major) of the epididymis. Its length is about twice the length of the left gonad in case 44, and slightly longer in case H-46. In the normal testis the head of the epididymis is a flattened disc which fits over the pointed anterior end. From the anterior end of this elongated lobe, an attenuated lobe,

reckoned as the body of the epididymis (*corpus epididymidis*), extends posteriorly and terminates in a conical mass, the tail of the epididymis (*globus minor*), which fits over the posterior end of the testis. From the tail of the epididymis the coiled vas deferens extends anteriorly. In the normal epididymis the epididymal body is a flattened, narrow band which connects the head and the conical tail of the epididymis.

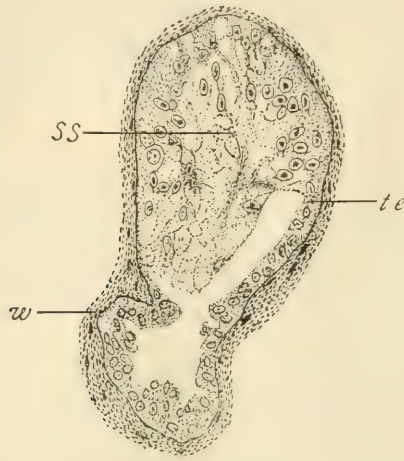


Fig. 16 The junction of a seminiferous tubule and a straight tubule (*tubulus rectus*) from the gonad of free-martin, H-37. *ss*, Sertoli-cell syncytium; *te*, abrupt beginning of epithelium of straight tubule; *w*, connective-tissue wall of tubule.  $\times 240$ .

Structurally, the epididymal tubules of the free-martin are well differentiated. The epithelium is usually simple, although in places it may appear two-layered. The cells are of the tall columnar type, except the few basal cells which appear more or less rounded. The former have on their inner surfaces long cilia. The epithelium rests upon a basement membrane, which is surrounded by a layer of smooth muscle fibers, which blend with the surrounding connective-tissue fibers (fig. 17, *mf*, *ee*). If comparison is made with the structure of the normal epididymal tubules (fig. 18, *mf*, *ee*), the resemblance is so close that it is difficult to distinguish the two.

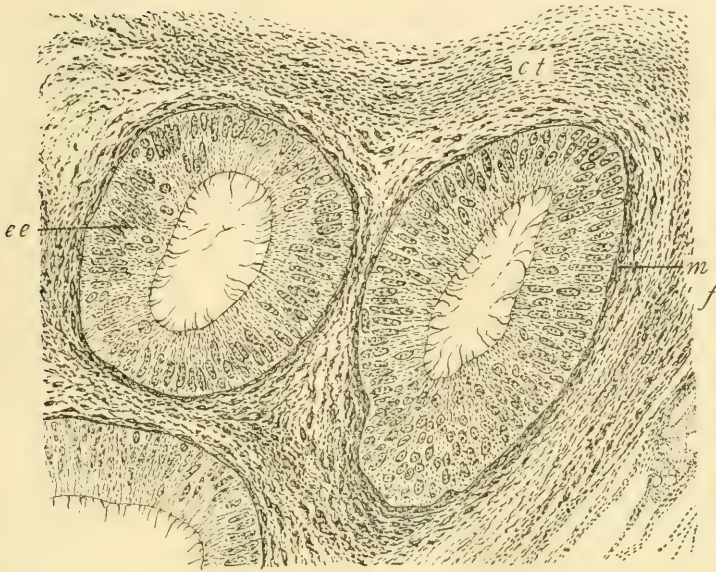


Fig. 17 Tubules from the head of the epididymis of free-martin, H-46. *ct*, connective tissue stroma; *ee*, ciliated, columnar epithelial cells; *mf*, layer of smooth muscle fibers.  $\times 307$ .

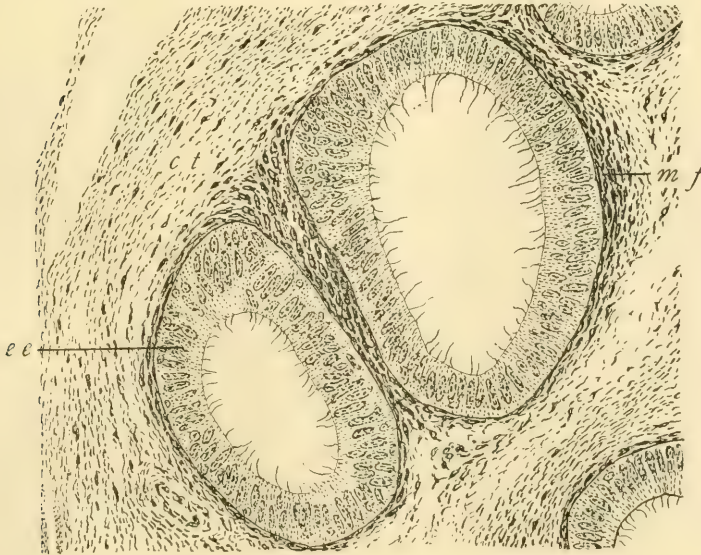


Fig. 18 Tubules from the head of the epididymis of a young male calf (six months?), Bos 12. *ct*, connective tissue stroma; *ee*, ciliated columnar epithelium; *mf*, layer of smooth muscle fibers.  $\times 307$ .



*The spermatic cord.* The gonads of specimens 44 and H-37 lie in peritoneal sacs, which are situated between the skin and the abdominal wall in the region of the groin. These peritoneal sacs are diverticula from the body cavity into which the gonads have descended (both the gonads and spermatic cords are retro-peritoneal, and not in the cavities of the peritoneal sacs; the peritoneal sac is merely wrapped around the gonad). In case 44 the cavity of the bulbular portion of the sac, which encloses the gonad, communicates with the rest of the coelom (body cavity) by a narrow canal. Along this channel is a passageway for the vas deferens, spermatic nerves, and blood-vessels. These three structures loosely bound together by connective tissue from the spermatic cord, as in the normal testis. Although the gonad of H-46 is not descended into a peritoneal sac, a more or less typical spermatic cord extends from its anterior end. Its vascular cord is well formed (7 mm. in diameter), and enters the anterior end of the gonad. Running parallel with the vascular cord and loosely bound to it by connective tissue is the vas deferens. In figure 10 the vascular cord (*vp*) is shown as a plexus of blood-vessels, which have thick fibromuscular walls of both circular and longitudinal fibers.

#### GENERAL DISCUSSION

In the free-martin a gonad with a primary female determination may transform into a structure which is morphologically a testis. The fundamental determining factor in such a transformation is the primary action of the male sex hormones. The sex hormones suppress the development of the ovarian cortex and stimulate the development of the other embryonic rudiments. That is to say, the sex hormones in no case cause the development of any new structure, as the primordium of each male structure developed in the free-martin gonad is present in the ovary at the time of sex differentiation. Whether sex hormones can stimulate the development of any new structure has not been demonstrated. However, if a gonad with a primary male determination were to transform into an ovary by the action of female sex hormones, a new structure, the ovarian

cortex would have to be added, since the male gonad never forms normally any homologue of the ovarian cortex.

Of considerable importance is the demonstration that a mammalian gonad with a primary female determination is capable of a graded series of transformations between an ovary and a testis. The exhibition of such a graded series in the free-martin undoubtedly corresponds to the variability in the time of introduction, the intensity, or the length of duration of action of the male sex hormones. That the time of introduction may vary is indicated by the variability in the anastomosis of the blood-vessels of the two embryos (Lillie, '17). According to this explanation, the gonads most completely transformed in the male direction must have had the most favorable conditions; the sex hormones were introduced early by an early strong anastomosis of the blood-vessels, or were unusually potent, or the duration of their action may have been prolonged. Obviously, if the conditions are less favorable, lower degrees of transformations will occur. It follows, therefore, that the gonads exhibiting a low degree of transformation must have had the least favorable conditions. To produce such gonads, the sex hormones must have had a late introduction, or were of low potency, or the duration of their action was shortened.

Such a graded series of transformations is shown by the relative sizes, shapes, and degrees of differentiation of the chief regions of the gonads and the associated epididymis. It has been shown in this paper that the sex-cord region exhibits definite and progressive steps in the male direction. The sex-cord region in the least transformed gonads is small and poorly organized, while in the most highly transformed gonads it is comparatively large, well organized, and differentiated. The sexual cords themselves exhibit a graded series of transformations between medullary cords and seminiferous tubules. The most highly transformed sexual cords are typical seminiferous tubules in every respect except that the transformation in the male direction has not proceeded to the production of any stage of male sex cells. The only exception to this rule is the brief and unconvincing statement of D. Berry Hart ('10), that in only one

gonad are spermatozoa present. His figure 1, plate II, shows seminiferous tubules, which in addition to the ordinary epithelium (Sertoli cells) show a few rounded cells which resemble in some respects primordial germ cells. No such picture of a seminiferous tubule of a free-martin showing either primordial germ cells or spermatozoa is shown in any of the specimens that I have examined. Certainly, such an important exception should have received more than brief mention.

In some of the foetal free-martin gonads a small proportion of the sexual cords enclose primordial germ cells, which, however, are destined to degenerate before birth. That these germ cells are distinctly female in character is shown by comparing them with the primordial germ cells of the medullary cords of normal foetal ovaries in cattle. As to origin, they are alike since the sexual cords of the free-martin gonad are medullary cords which are transforming into seminiferous tubules. The fate of the primordial germ cells is the same in both the free-martin gonad and the normal foetal ovary. The medullary cords with their germ cells degenerate and disappear before birth in the normal ovary of cattle, the first to disappear being the germ cells. Allen ('04) has described a similar fate of the primordial germ cells found in the medullary cords of rabbit and pig ovaries. It would appear that this phenomenon is the regular event in the development of the mammalian ovary. Since these primordial cells in the sexual cords of the free-martin gonad and in the medullary cords of the normal foetal ovary are identical as to origin, structure, degeneration changes, and disappearance before birth, it seems reasonable to conclude that the primordial germ cells of foetal free-martin gonads are female in type.

As the sex-cord region shows more pronounced male organization, the rete region simultaneously makes progress in the male direction in several particulars. In the first place, its primitive position at the hilus of the gonad is more or less lost by the growth of the sex-cord region around its sides. However, the growth of the sex-cord region is not sufficient even in the most completely transformed gonads to bring the rete into the center of a cylindrical gonad as it is in the normal testis (fig. 1, D and



E), but it lies eccentric to the greater portion of the sex-cord region. Secondly, as a rule, the distance of the penetration of the core of rete tubules into the gonad varies directly with the increase of maleness (fig. 1, B, C, and D). Thirdly, the lumina of the rete tubules increase in size as the gonad increases in maleness. In the fourth place, the rete tubules establish connections with the vasa efferentia and with the seminiferous tubules; only a few such connections are made in the moderately transformed gonads, while in the most highly transformed gonads many are made. Obviously, the rete of the free-martin gonad may be transformed into a typical rete testis.

The connective-tissue capsules exhibit three more or less distinct and progressive stages of development in the male direction. These stages correspond only in a very rough way to the degree of transformation of the gonads, but correspond very closely to the stages passed through in the normal development of the tunica albuginea of the testis. The first stage is characterized by a comparatively thin layer of connective-tissue fibers; in the second stage the capsule possesses two layers, the tunica albuginea and the tunica vasculosa, while in the third stage the tunica vasculosa is merged with the tunica albuginea.

Closely associated with the degree of development of the connective-tissue capsule is the distribution of the blood-vessels. In the highly transformed gonads a vascular cord, which is typically like the normal vascular cord of the testis, penetrates into the anterior end to connect with the tunica vasculosa or with the vascular portion of the tunica albuginea. In less transformed cases a much less typical vascular cord enters the gonad at a point slightly posterior to the anterior end, from which its blood-vessels are distributed in some specimens to the tunica vasculosa, and in others directly outward to the sex-cord region as in the normal ovary.

The epididymis also exhibits a graded series of transformations in the male direction. In the least transformed gonads there are no traces of epididymal tubules; in the moderately transformed gonads there are traces of tubules, while in the most highly transformed gonads the typical epididymal bodies are present.

A question of considerable importance to the problem of sex-differentiation in mammals is to determine the limit of transformation of an ovary in the male direction by the action of sex hormones. The most extreme case is the gonad of H-37. Although the gonad (left) has attained a comparatively large size, it is nearly twenty times smaller than a normal testis of approximately the same age. Structurally, it is a typical testis in every respect except that all stages of the male germ cells are absent. So complete is the transformation that some doubt may arise as to its classification as a free-martin gonad. However, the following facts indicate without a reasonable doubt that the gonads are from a free-martin: 1) The specimen in question was born co-twin to a normal bull. 2) The external genitalia are predominately female, although the clitoris is partially transformed into a perforated penis. Numan ('43) figures in his plate XI (refigured by Lillie, '17, fig. 29) the reproductive system of a free-martin, which shows the external genitalia transformed in a manner almost identical with that of case H-37. 3) The gonad of this specimen forms a member of a graded series of transformations in the male direction. 4) The internal reproductive system, aside from the gonads, is in all essentials of the usual free-martin type, except there is a greater progression in the male direction. 5) The possibility that the gonads are from a cryptorchid male is dismissed by the fact that in all cryptorchids the external genitalia are normal. Furthermore, cryptorchidism in cattle has not been reported. Obviously, then, an ovary under the influence of male sex hormones may transform into a testis which is morphologically complete, but which is functionally inactive so far as the production of germ cells is concerned (the interstitial cells may be physiologically active).

Such a reproductive gland approaches very closely, if not actually reaches the limit of transformation in the male direction by hormonal action, a condition which indicates that the optimum conditions for the transformation of an ovary were fairly closely realized in this case. What are the optimum conditions? The optimum conditions, so far as the primary cause is con-

cerned, have already been considered in the first part of the general discussion as due to an early anastomosis of the blood-vessels between the different-sexed twins, and therefore an early introduction of the male sex hormones or the sex hormones may have been unusually potent or the duration of their action may have been prolonged. These factors are responsible at least for the early changes in the transformation of the gonad. How much of the subsequent events is due to the initial stimulus of the sex hormones and how much is due to other factors is difficult to determine. However, a few suggestions as to the factors responsible for the later changes may be considered. In the first place, the sex hormones may continue to act until birth, providing the vascular connections persist (the foetal membranes are still united at birth, according to Tandler and Keller, '11). This necessarily involves the further assumptions that the interstitial cells of the testis of the male twin are active throughout foetal life or at least periodically active, and that these cells produce sex hormones. Secondly, owing to the absence of the cortex, it is difficult to state how much of the male development may be the result of the absence of normal ovarian secretions. It is known that in the absence of such secretions male characters tend to develop. Pearl and Surface ('15) described a cow with cystic degeneration of the ovaries which led to the development of male secondary sexual characters. Spayed females in birds take on male secondary sexual characters, (Goodale, '16), and the castration of the hen-feathered Sebright male results in the development of the plumage characteristic of the cock-bird (Boring and Morgan, '18, Morgan, '19). In the third place, the interstitial cells of the free-martin gonad may furnish sex hormones which aid in its own differentiation. Certainly, some relation exists between the high degree of maleness and the hypertrophy of the interstitial cells, as in all of the most highly transformed gonads they are abundant. If these cells are to play any part in differentiation of a gonad, they would be expected to occur at least in some of the foetal stages. This is not the case, at least in the early foetal stages, as no traces have been found.



Just how much growth and differentiation of the free-martin gonad occur after birth has not been determined, owing to the difficulty of obtaining the late foetal stages. In the early free-martin embryos examined (oldest embryo was 28 cm. long), the gonad is smaller than normal and the seminal vesicles have not yet appeared. In the normal male the seminal vesicles are well indicated from 16.8 cm. on. In approximately 80 per cent of the post-natal free-martins the seminal vesicles are well-defined structures. In some the prostate appears (H-18) (cf. literature). It would appear, therefore, that there is a delay in growth during the late prenatal stages. With this gap filled, it would be possible to make a more careful analysis of the factors which are responsible for the later steps in the modification of the reproductive system. At birth the possibility of any further action of the male sex hormones terminates. During this period of late foetal development, search should be made for any evidence of interstitial cell activity in the testis of the male twin, and also to determine the degree of anastomosis of the extra-embryonic blood-vessels between the male and female individuals.

A parallelism is seen between the degree of transformation of the gonad and the degree of transformation of other parts of the internal reproductive system, the external genitalia and the phenomenon of descent. In the case of the reproductive-duct system the parallelism is not distinct, but in general there is a tendency for the female parts to disappear and for the male parts to develop as the gonads are transformed in the male direction. The external genitalia are the least liable to undergo modification, but in cases where the normal male limit is closely approached, they may also be transformed. That is, in cases where the reproductive glands are structurally typical testes, and have descended into the groin region, the external genitalia assume male characters (the clitoris in H-37 is partially transformed into a penis). Obviously, the external genitalia, the system of ducts and accessory reproductive glands as well as the gonads exhibit a graded series of transformations in the male direction.

Not only is there this general correlation between the various parts of the free-martin reproductive system, but the correlation is still closer if the parts on one side are compared. Case 44 (table 2) shows a large left gonad associated with a large Wolffian duct and a large seminal vesicle, while the right gonad has a smaller duct and a smaller seminal vesicle. Case H-37 shows the left gonad one and a half times larger than the right gonad, and the left seminal vesicle is fairly well developed and possesses a duct which ends blindly, while the right seminal vesicle is poorly developed and its duct entirely absent. Lillie ('17) showed that disturbances in relation of parts in foetal stages are found on the right side, as, for instance, in his case 2 (fig. 20, A) where the gubernaculum on the right side evaginates into the body cavity instead of into the body wall, as the left one does. These lateral variations certainly indicate that physiological factors, other than the action of sex hormones, play a part in correlation. There is some embryological basis for these lateral variations, as is seen from an examination of a number of ovaries, the majority of which are from young calves. In the number examined (20) about 65 per cent show the left ovary to be larger and the follicles more advanced than in the right.

A very interesting situation is presented if the lateral variations of the reproductive glands of the free-martin are compared with similar variations in other vertebrates. According to Simpson ('36), in the human the left side usually has the female type of gonad and disturbances are usually found on the right side (a number of more recent cases support this observation). Among other groups of vertebrates, lateral variations occur normally where the left ovary is larger and the functional one. The monotremes have a larger left ovary and eggs have been found only in the left oviduct. In birds the right ovary degenerates and among certain of the skates (*Trygon*, *Myliobatis* and relatives) the left ovary alone functions. Although there may be certain mechanical factors which result in the functioning of one ovary, it is certainly no coincidence that it is always the ovary on the left side which is functional. From the above considerations it would appear that some physiological processes

underlie these asymmetrical disturbances. The blood supply may be greater to the left gonad than to the right, yet this cannot be regarded as the primary factor.

The interrelation between the reproductive glands and other glands of internal secretion is supported by the observations of many, yet none of these observations furnish any definite clue as to the nature of the correlation. The whole developmental history of the free-martin reproductive system is certainly complicated by such correlations as these.

If the intersexual condition in the free-martin gonads is compared with the condition of intersexuality in the gonads of other animals, definite similarities in structure and origin are recognized. Goldschmidt ('12, '14, '16) has shown that both the male and female individuals of *Lymantria* contain the primordia for each sex. Which sex is to appear depends upon the quantitative relations of both male and female sex factors of the gametes. If the crosses between two species are made in such a way that the male factors have dominance over the female factors, an ovary transforms into a testis. Thus with a variable quantity of the male factors all degrees of transformations are possible. On this point Goldschmidt ('16, p. 713) states: "This is a body [sex gland] looking externally like a testis, but showing in sections every single step between an ovary with nothing but immature eggs through a mixture of ovarian and testis tissue to a real testis." The reverse changes, that is, the transformation of a testis into an ovary, are also possible. It has been shown that the free-martin gonad is primarily an ovary, which would be due, according to the conceptions of Goldschmidt, to the dominance of the female factors over the male. Lillie ('17) pointed out that the intersexual condition in the free-martin gonad may be explained "as due to an acceleration or intensification of the male factors of the female zygote by the male hormones." The degree of transformation would be dependent on the quantity of the hormone. Obviously, in the free-martin gonad the male-sex expression is the result of a variable quantity of internal secretions, instead of a variable quantity of male-sex factors as in the gonads of the intersexual hybrids of the gypsy-



moth. Goldschmidt ('17), in comparing the hormonal intersexuality of the free-martin with the zygotal intersexuality of Lymantria, attempts to point out that the direction of sexual differentiation changes at the time when specific sex-determining hormones can act. In the free-martin he supposes that specific sex-determining hormones arise in the interstitial cells, and act the moment they enter the blood of the female, while in Lymantria they arise from every single cell of the body.

Among mammals in general an intersexual condition of the gonad is somewhat rare. As a rule, in the cases that have been examined, the gonad is usually part ovary and part testis (Pick, '14). Gudernatsch ('11) points out that hermaphroditism in the sense of separate ovaries and testes has not been demonstrated in man or even in other mammals beyond a doubt. Furthermore, in no case does the testicular part bear any stage of male germ cells, but the ovarian part may produce germ cells which are at least structurally normal. The possibility of the formation of such intersexual gonads in mammals by the action of sex hormones is suggested by Lillie ('17). This possibility is made still stronger by the study of one of the free-martin gonads (case H-42). This gonad is partly ovary and partly testis. The ovarian part contains distinct follicles with germ cells which are apparently normal, while the testicular part contains typical seminiferous tubules, but the germ cells are absent. Magnusson ('18), reports free-martin gonads which have a similar structure with the exception that the primary follicles lack germ cells. This ovarian part, which is apparently an exceptional condition in the free-martin gonad, probably arises either as a consequence of a late introduction or of a temporary action of the male sex hormones. In the former the cords of Pflüger invaginate to a slight extent, while in the latter alternative the ovary was only partly transformed into a testis. Since the intersexual condition of the free-martin gonad (case H-42) resembles the intersexual conditions of the gonads of other mammals, it appears that they may have an interpretation in common. It thus follows that in all mammals where there is a possibility of embryonic anastomosis of the blood-vessels between individuals of opposite sexes,

the gonads may be intersexual. The rather frequent occurrence of intersexual gonads in the pig may have some such explanation as this. The appearance of intersexual gonads in mammals, which, as a rule, furnish no possibilities of the intermingling of sex hormones through the fusion of foetal membranes, must be interpreted in some other way. In such cases it is not impossible that disturbances in the normal relationship of the maternal blood supply to the foetal blood may account for the intersexual condition of the gonads.

The definite transformation of an ovary into a testis raises the question of the existing morphological homologies between the two reproductive glands. As early as 1870, Waldeyer, in his classic book, "Eierstock und Ei," suggested that the potentialities for the development of both ovary and testis existed in the same individual, but he erred in considering that the male and female reproductive glands arise from different portions of the genital ridge. From a study of the adult gonads of the bat, Van Beneden ('80) formulated the hypothesis that the testis and ovary possess morphological structures in common. He homologized medullary cords with seminiferous tubules, medullary tubes with straight tubules, and the rete ovarii with the rete testis. Such a homology as suggested was not fully justified until the comparative studies on the development of the ovary and testis were made by various investigators. Allen ('04) showed for the rabbit and pig that the first set of sexual cords (medullary cords) of the ovary and of the seminiferous tubules of the testis are homologous structures, which are both formed as tubular invaginations of the epithelium of the genital ridge. Simultaneously with the development of the first set of sexual cords, the rete cords of both ovary and testis arise in the same manner from the epithelium of the anterior end of the genital ridge. A zone of connective tissue, the primary tunica albuginea, which lies between the medullary cords and the cords of Pflüger, is homologous with the tunica albuginea of the testis.

In man (Felix, '11) and in the cat (Kingsbury, '13) the morphological homology of the medullary cords of the ovary and the seminiferous tubules of the testis appears difficult to establish

with certainty, owing to the absence in the ovary of a more or less distinct line of demarkation between an inner epithelial mass (medullary cords) and an outer epithelial mass (cords of Pflüger). Although the separation of the two regions is not obvious in the late foetal stages of the cat's ovary, these regions are distinct in the earlier stages, owing to the growth of a distinct primary tunica albuginea between the two (Sainmont, '05). In the ovary of both the cat and man the sexual cords of the medulla take no part in the formation of ova in the fully developed ovary, and in this respect resemble other mammalian ovaries, where the homology has not been questioned (as in cattle; fig. 3, *ta*). Furthermore, in both cases occurs the rete ovarii, the undoubted homologue of the rete testis. These facts are certainly sufficient to indicate that the ovarian-medulla-testis homology applies in the cat and man.

Fortunately, in the free-martin, nature has performed a veritable crucial experiment, which demonstrates without question that the ovary possesses structures which have their morphological equivalents in the testis. That they are morphologically equivalent is shown by the transformation of certain ovarian structures into testicular structures under the influence of sex hormones. The following homologies are established: 1) The medullary cords precisely and definitely transform into seminiferous tubules. 2) The rete ovarii transforms into a rete testis chiefly by developing connections with the seminiferous tubules and with the vasa efferentia. 3) The primitive tunica albuginea of the ovary becomes the tunica albuginea of the free-martin gonad. 4) Associated with the embryonic ovary is an epididymal primordium which transforms into a typical epididymis.

If a comparison of the sex-cord region of the free-martin is made with certain well-known changes in the sex-cord regions of mammalian testes, marked structural similarity is seen. In both, the interstitial cells are hypertrophied and each seminiferous tubule has a thickened wall within which is a Sertoli-cell syncytium free from germ cells. Such changes are present in cryptorchid testes (Whitehead, '08, and Hanes, '11), and are experimentally produced in the following ways: exposure to



X-rays (Regaud, '10), feeding a diet deficient in water-soluble vitamine (Allen, '19), eliminating the sympathetic nerve supply (Kuntz, '19), transplantations (Moore, '19), etc.

These cases (at least the experimental ones) all indicate that the atrophy of the male germ cells is accompanied by the hypertrophy of the interstitial cells. In the free-martin gonad there is no atrophy of the male germ cells to accompany the hypertrophy of the interstitial cells. It would therefore appear that the increase of the interstitial cells is not necessarily associated with the phenomenon of the degeneration of the germinal cells. There may be some more fundamental changes in metabolism, which are responsible for the observed modifications. There is some evidence that metabolic changes result in certain structural modifications in the testis, as, for instance, the cyclic changes in hibernating animals (Rasmussen, '17 and '18). It is problematical whether changes in metabolism bring about the degeneration of germ cells or the absence of germ cells brings about changes in metabolism.

A question of considerable interest arises as to the relation of the interstitial cells to the sexual instincts and to the secondary sexual characters in the free-martin. In mammals having cryptorchid testes the typical sexual instincts are manifested and are apparently associated with the functioning of the abundant interstitial cells (Hanes, '11; Whitehead, '08). Since the structure of the sex-cord region of the free-martin gonad resembles in essential details the sex-cord region of a cryptorchid testis, it might be expected that the free-martin would have the usual sexual instincts of a male. This is apparently not the case, at least so far as the literature discloses. According to the accounts of Lundberg ('64), Zschokke ('00), Hunter ('86) and Magnusson ('18), free-martins fail to exhibit sexual instincts. Hart (10) states concerning one of Hunter's free-martins on page 231: "This animal was seven years old; went with the cows and bull; never showed any desire for either." Zschokke states that in the Alps the free-martin is used like the ox as a beast of burden, because it is strong and tame, or it is fattened for the market.

Another characteristic of the cryptorchid male is the normal appearance of the secondary sexual characters. The free-martin, however, according to the observations of Magnusson ('18), Pusch ('11), Zschokke ('00) and others, resembles a castrated male in body form, development of the horns, etc. Hunter ('86, p. 53) regarded it more like the ox or spayed heifer than the bull or cow. Tandler and Keller ('10), who performed elaborate experiments on the influence of castration on the body form of the male and female in cattle, conclude that both male and female converge to a 'common form' (asexual). They regarded the free-martin as resembling more closely the castrated female in form. Hart ('10) figures one of Hunter's free-martins which shows very distinctly the head and horns of an ox or spayed female, and the fore and hind quarters distinctly male in type. It would appear from these observations that the interstitial cells in the free-martin gonad fail to play any strong rôle in the production of secondary sexual characters.

SPECIMEN		GONAD			
Number	Age	Size	Tunica albuginea	Sexual cords	Intertubular tissue
66	5 days	About 5 mm. long. 2 mm. wide	Outer layer of compact fibers; inner layer barely indicated and constitutes a tunica vasculosa (few blood vessels)	Masses of supporting epithelial cells; sexual cords—some resemble medullary cords, others resemble seminiferous tubules; and still others showing transitional stages between the two types. Germ cells absent	Few interstitial cells
42	21 days	13.5 mm. long. 5 x 3 mm. in cross-section	As in case 66	As in case 66	As in case 66
H-18 Fig. 5	31 days	4 x 6 mm. in cross-section	Outer layer compact, comparatively thick. No tunica vasculosa. Blood-vessels enter hilus, and are distributed outwardly	Masses of supporting epithelial cells very few. Sexual cords as in case 66. No germ cells	As in case 66, except more easily recognized
H-46 Fig. 10	5½ wks.	8 x 10 mm.	Capsule increased in thickness. Outer layer of compactly arranged fibers; inner layer loosely arranged, and enclosing blood-vessels, and constitutes a tunica vasculosa	Majority of sexual cords resemble seminiferous tubules of young male calves (figs. 12 and 8). Nuclei of supporting epithelial syncytium arranged in a single layer at periphery, and from inner ends extend strands of cytoplasm to the center. No germ cells	Connective-tissue stroma abundant. Interstitial cells moderately numerous
44	7 wks.	Left 35 x 25 mm. Right 13 x 15 mm. (Measurements include epididymis)	Outer layer distinct, thick. Tunica vasculosa only well-developed in places	As in H-46	As in H-46
H-37	14 mos.	Left 60 x 40 mm. Right 40 x 25 mm.	Tunica vasculosa is intimately merged with the tunica albuginea. In the inner portion of the latter are blood-vessels	Seminiferous tubules typically like normal tubules, except germ cells are absent. A Sertoli-cell syncytium structurally identical with the syncytium of adult testes. Wall thickened	Interstitial cells much hypertrophied
H-42	18 mos.	Left 12 x 4 mm. Right ? x 4 mm.	Outer layer of more compactly arranged fibers; inner layer of loosely arranged fibers, enclosing blood-vessels, constituting a tunica vasculosa	Two kinds of sexual cords, those which resemble seminiferous tubules, and those which resemble primary follicles. A few egg cells within the follicles	Many interstitial cells; two types, those resembling the interstitial cells of normal testis, and those resembling lutein cells of a corpus luteum
H-36	2.5-3 yrs.	20 x 10 mm.	As in H-42	Very few sexual cords, which resemble seminiferous tubules. No germ cells	No interstitial cells found
H-40	3 yrs.	10 x 4 mm.	Capsule much thickened. Blood-vessels scattered. Fig. 2	Sexual cords few; some resemble small seminiferous tubules, other medullary cords. No sex cells	Interstitial cells moderately abundant



GONAD					
Tubuli recti	Rete tubules	Epididymis	Vascular plexus	Seminal vesicles	External genitalia
Few	Eccentrically placed; extends over half way to posterior end	Mere traces of tubules	Flattened cord of blood-vessels enters anterior end of gonads	Not examined	Typically female
Few	As in 66	Rete established connections with four vasa efferentia tubules (fig. 9)	As in 66, fig. 9	Not examined	Typically female
Few	As in 66	Absent	No definite vascular cord as in 66 and 42. Few blood - vessels enter hilus at anterior end	Two small masses having imper-vious ducts. (A prostate gland is present)	Typically female
Moderately numerous	Penetrates to posterior end of gonad; eccentrically placed (fig. 10). Toward posterior end sex-cord region completely surrounds rete	Rete connects with head of epididymis at anterior end; tail of epididymis attached at posterior end of gonad—connected with head by the body of the epididymis	A vascular cord (7 mm. in diameter) enters anterior end of gonad (fig. 10). Vascular cord and vas deferens loosely united by connective tissue forming spermatic cord	No seminal vesicles	Typically female
As in H-46	Penetrates about half the length of gonad; eccentrically placed	As in H-46	As in H-46	Two long vesicles	Typically female
Very numerous. Fig. 16	Extent not examined, but probably extends to posterior end. Sex-cord tissue lies between rete and tunica albuginea	As in H-46	As in H-46	Right vesicle less developed than left. Left duct ends blindly; right duct absent	Clitoris transformed into a penis-like structure, otherwise the external genitalia are female
Few	Extends to posterior end of sex-cord region; eccentric to sex-cord region	Rete makes connections with several epididymal tubules	Not examined. No spermatic cord according to Cole	Vesicle (4 cm. long). Ducts impervious	Typically female
Absent	Extent not determined	Absent	Very small plexus of blood-vessels attached at hilus	Two lobulated vesicles, attached to vas deferens	Typically female
Absent	Extend two-thirds of the length of the sex-cord region	Absent	Plexus of blood-vessels emerge from anterior end of each gonad	Two vesicles communicating with vas deferens	Typically female

SPECIMEN		OVARY			
Number	Length, age	Size	Tunica albuginea	Medullary cords	Cords of Pflüger
N 14	6.3 cm.		Primary albuginea a distinct layer	First set sex cords numerous; no well-defined walls to cords. Supporting epithelial cells irregularly arranged and closely packed together	Short ingrowths continuous with germinal epithelium
N 7	8.3 cm.		As in N 14	As in N 14. Few primordial germ cells	Short ingrowths continuous with germinal epithelium; little differentiated from it
N 40	10.2 cm.		More distinct and wider	As in N 7	As in N 7
N 26	14.0 cm.	3.4 mm. long	Primary albuginea as in N 40. Definitive albuginea barely indicated	Cords contain germ cells, forming "medullary follicles"	Cortex relatively thicker. Cords distinctly separated from each other by strands of connective tissue
N 23	17.0 cm.	4.39 mm. long	Primary albuginea is wider. Definitive albuginea as in N 26	Medullary primary follicles containing germ cells. Nuclei of supporting epithelial cells more or less irregularly arranged, sometimes forming a single layer with strands of cytoplasm extending centrally	Cords still attached to germinal epithelium. At inner ends of cords are epithelial nests (primary follicles)
N 15	18 cm.		As in N 23	As in N 23. In addition to the sexual cords, occur masses of unorganized supporting epithelial cells	As in N 23
N 24	20 cm.	4.44 mm. long	Primary albuginea is less distinct owing to its mergence with connective tissue of medulla	Medullary cords fewer than N 15, some degenerating. Connective tissue increased in medullary region	Primary follicles at inner ends of cords more numerous
N 25	23 cm.	6.83 mm. long	As in N 24	As in N 24. Some medullary cords having nuclei of supporting epithelial syncytium arranged more or less into layers	As in N 24
N 20	29.5 cm.	7 mm.	As in N 24 except the definitive albuginea is thicker	Medullary cords rather numerous, and surrounded by a rather dense stroma of connective tissue	Primary follicles numerous
Bos 8	56 cm.	8 x 4 mm.	Primary albuginea a distinct and wide layer (fig. 3, <i>ta</i> ). Very thin definitive albuginea	Few degenerating cords (fig. 3, <i>mc</i> )	Very numerous (fig. 3, <i>p</i> )
Bos 9	106.5 cm. (mature foetus)	12 x 5 mm.	Primary albuginea no longer a distinct layer, but is continuous with the stroma of the medulla and of the cortex. Definitive albuginea thin	Very few medullary cords found. Medulla very vascular	Many follicles enlarged—granulosa cells hypertrophy filling follicular cavity—germ cells degenerating, resulting in large follicular masses of granulosa cells
Bos 6	Young calf 6 mos. old ?	Left 25 x 17 mm. Right 25 x 9 mm.	Definitive albuginea very thin and inconspicuous	No medullary cords. Medulla comparatively small and composed of a stroma of dense connective-tissue fibers, between which are numerous blood-vessels	Same as Bos 9, except that the degenerating follicles may become much larger (atretic follicles)
Bos 1	Adult	30 x 12 mm.	Definitive albuginea, a definite layer of densely arranged connective tissue fibers	No medullary cords	Normal follicles in cortex. One large corpus luteum

OVARY				
Stroma	Tubuli recti	Rete tubules	Epoöphoron	Vascular plexus
Little connective tissue fibers	None	Enters anterior end at hilus, but does not penetrate far posteriorly. Lumina forming	Rete tubules make connections with tubules of Wolffian body	Blood - vessels enter hilus along with rete
No interstitial cells	None	As in N 14	Slight degeneration of tubules of Wolffian body	As in N 14
Scanty stroma; no interstitial cells	None	Extends only a short distance posteriorly	As in N 7	As in N 14
Few cells in stroma resembling interstitial cells	Absent	Extends about one-half the length of the medullary cord region. Enters anterior end at hilus	Wolffian body tubules growing smaller	Blood - vessels enter hilus as in the above cases
No interstitial cells found	Absent	Enters anterior end; extent one-half the length of medullary-cord region. Rete smaller, and extent posteriorly less than in testis from embryo of same age	Wolffian body tubules still smaller and fewer than in N 26	As in N 26
Stroma abundant	Absent	As in N 23	As in N 23	As in N 26
No interstitial cells observed	None	Extent about one-half the length of the medullary cord region. Smaller than rete of testis from embryo of same age	Wolffian body mostly degenerated. Tubules of epoöphoron and paroöphoron present. Rete connects with epoöphoron	As in N 26
As in N 24	None	As in N 24	As in N 24	As in N 24
As in N 24	None. The rete tubules and medullary cords closely approximated	Enters hilus, extends one-half the length of medulla. Diameter less than rete testis of N 19	As in N 24	As in N 24
No interstitial cells recognized. Stroma abundant	Absent, although some medullary cords lie close to rete tubules	Lies at hilus; projects to posterior end of medullary-cord region	?	A flattened cord of blood - vessels enter hilus at anterior end
Occasional cells in medulla stroma which resemble interstitial cells	Absent	Distinct tubules with lumina. Penetrates almost to posterior end of medullary cord region	?	A cord of convoluted blood - vessels enter the anterior end at the hilus, along with the rete tubules
?	Absent	Traces of a few degenerating tubules at hilus	?	As in Bos 9
?	Absent	Absent	Absent	As in Bos 9



SPECIMEN		TESTIS		
Number	Length, Age	Size	Tunica albuginea	Seminiferous tubules
N 13	4.8 cm.		Outer layer of compact connective-tissue fibers. Inner layer of loose fibers, which enclose blood-vessels	Solid cords of compactly and irregularly arranged supporting epithelial cells. Cord walls faintly indicated. Primordial germ cells if present, are not evident
N 10	7.0 cm.	3.03 mm. long	Thin. Fibers more compactly arranged in outer layer. Tunica vasculosa as in N 13	Cords more definite, little branching of cords. Supporting epithelial cells formed into a syncytium; nuclei irregularly arranged into two and three layers. Primordial germ cells present
T 19	8.0 cm.	3.5 mm. long	As in N 10	Seminiferous tubules increasing in size, and looser in structure. More regularity in arrangement of nuclei of supporting epithelium. Cytoplasmic strands from inner ends of nuclei extend toward potential lumen, are just beginning to develop. Primordial germ cells
T 16	12.75 cm.	4.45 mm. long	As in N 10. Outer layer more compact	Nuclei of supporting epithelial cells mostly arranged into a single layer; in some two layers. Cytoplasmic strands more prominent. Few distinct primordial germ cells
T 6	16.8 cm.	4.86 mm. long	Wider, otherwise as in T 16	Tubules larger, looser. All nuclei of syncytium in a single layer, except a few scattered ones. Distinct cytoplasmic strands extending inwardly from nuclei. Few primordial germ cells
N 21	20.0 cm.	4.04 mm. long	Both layers distinct. Fig. 4	Nuclei of supporting cells as in T 6, except closer together. Tubules more branched at periphery of testis. See fig. 4
T 4	24.0 cm.	8.67 mm. long	Distinction between outer and inner layers not as well marked at this stage	Same as N 21
N 19	31.0 cm.	8.26 mm. long	Two layers still distinct, but connective tissue is increasing in compactness in tunica vasculosa	Nuclei of supporting epithelium lie closer together in the single layer—cytoplasmic strands still more pronounced. Few primordial germ cells
Bos 10	61.0 cm.	25.0 mm. long. 8.0 mm. wide	Connective tissue nearly as compact in tunica vasculosa as in the outer layer	As in N 19
Bos 3	89.0 cm. (Mature fetus)	36.0 mm. x 11.0 mm.	Two layers merged into one—inner portion of which contains the blood-vessels 1.5 mm. thick	Same as N 19, except there is more regularity in the arrangement of the nuclei of the supporting epithelial cells, and the cytoplasmic strands are more pronounced. Few primordial germ cells. Fig. 7
H-45	2 days	?	Not examined	As in Bos 3
H-26	12 days	?	Not examined	As in Bos 3
Bos 12	Young calf 6 mos. ?	40.0 mm. x 12.0 mm.	As in Bos 3, 1.25 mm. thick	As in Bos 3, except the nuclei of the syncytium are more closely approximated. Fig. 8
Bos 2	Adult	125.0 mm. x 57.0 mm.	As in Bos 3 except thinner 1 mm. thick	The strands of cytoplasm of supporting epithelial cells (Sertoli cells) have become resolved into a net-work of strands, between which are the multiplying male sex cells. Nuclei of Sertoli cells usually lie near periphery of tubules, but in no such regularity as in Bos 12. Fig. 13

TESTIS

Intertubular tissue	Tubuli recti	Rete tubules	Epididymis	Vascular plexus
Very little stroma of connective tissue	None found, although rete and seminiferous tubules are approximated	Enter anterior end; penetrate a very short distance posteriorly. Lies at hilus. Lumina barely indicated	Rete tubules establish connections with renal corpuscles of Wolffian body at anterior end of testis	Blood-vessels enter at hilus, thence to tunica vasculosa
Connective-tissue stroma scanty	Branches of rete tubules make connections with seminiferous tubules	Projects about two-thirds of length of testis. Lies nearly in center of round gland. Several distinct lumina	Large Wolffian body. Connections made as in N 13	Enter along with rete. Distribution as in N 13
As in N 10	More distinct	Extends farther into testis than N 10. Still a little eccentric to the center. Many distinct lumina	As in N 10	As in N 10
As in N 10	As in T 19	Extends entire length of testis. Center of round testis. Lumina more numerous	Large and well developed	As in N 10
Moderate amount of stroma. Few interstitial cells	Many	Tubules large in diameter; much branched. Extend entire length. A central axis	As in T 16	A distinct vascular plexus (cord-like) enters anterior end of testis
Stroma abundant. Interstitial cells many	Many. Fig. 4	Extends to posterior end. Central core shown in fig. 4	As in T 16	Blood-vessels in vascular cord more convoluted and larger
As in N 21	As in N 21	As in N 21	As in N 21	Vascular cord structurally is a network of venous spaces and arterial vessels
Interstitial cells numerous	As in N 21	As in N 21	Not examined	Not examined
Interstitial cells numerous	As in N 21	As in N 21	Globus major, globus minor, and body of epididymis well developed	As in T 4 except larger in diameter
Interstitial cells moderately numerous	Present. Fig. 7	Central core, extending to posterior end as in N 21	As in Bos 10	As in Bos 10, only larger
As in Bos 3	As in Bos 3	Present	Not examined	Not examined
As in Bos 3	As in Bos 3	Present	Not examined	Not examined
Interstitial cells numerous. Fig. 8	Numerous connections	As in Bos 3. Fig. 14	Globus major a disc-like cap fitting over anterior end of testis, and connected with rete. Globus minor, a conical mass attached to posterior end of testis. Connecting these two parts is the strap-shaped body of the epididymis. Fig. 18	Vascular cord 5 mm. in diameter enters anterior end, just at one side of the connection of rete and epididymis
Stroma and interstitial cells moderately numerous	Many. Fig. 13	As in Bos 3	As in Bos 12, only much larger	As in Bos 12, except larger; 12 mm. in diameter

## SUMMARY

1. In the free-martin an indifferent gonad with a primary female determination transforms in the male direction under the influence of male sex hormones.

2. Three distinct steps may be recognized, which may be characterized as low, medium and high degrees of transformation in the male direction. These three groups constitute a chain of connecting links between an embryonic ovary and a testis.

Such a graded series of transformations is evidenced as follows:

*a.* The sexual cords exhibit a series of gradations between medullary cords and seminiferous tubules.

*b.* The interstitial cells increase in number as the gonad transforms in the male direction.

*c.* The rete transforms in the male direction by developing connections (tubuli recti) between the rete tubules and the seminiferous tubules, and by connections between the rete tubules and the epididymal tubules. Such connections are not made in the gonad exhibiting a low degree of transformation. In the group of moderately transformed gonads a few such connections are made, while in the most highly transformed gonads such connections are numerous.

The distance that the rete penetrates into the sex-cord region varies according to the degree of transformation of the gonad. In the most highly transformed gonad it penetrates to the posterior end of the sex-cord region, as in the normal testis.

The rete becomes less eccentrically located with reference to the sex-cord region in cases of pronounced male organization. It is never centrally placed as in the normal testis.

*d.* The epididymis is absent from gonads exhibiting a low degree of transformation; only the head of the epididymis is present in gonads showing a moderate degree of transformation, while a typical epididymis is present in the most highly transformed sex glands.

*e.* The distribution of the blood-vessels correspondingly ranges from a typical ovarian arrangement to a typical male arrangement.



3. The limit of the transformation in the male direction by hormonal action is represented by a testis which is morphologically complete, but functionally inactive so far as the production of germ cells is concerned.

4. The exhibition of a graded series of transformations between an ovary and a testis apparently corresponds to, 1) the variability in the time of the introduction of the male sex-hormones; 2) the potency of the hormones, or, 3) the duration of the hormonal action. These are the primary and fundamental determining factors. Other factors may be responsible for subsequent events in the transformation, as, 1) absence of normal ovarian secretions and, 2) secretions from the interstitial cells of the free-martin gonad.

5. The degree of transformation of the gonads is correlated in a rough way with the degree of transformation of other genital organs (vas deferens, seminal vesicles, uterus, and external genitalia). These correlations are closer between the genital organs of one side than between opposite sides.

6. The hypertrophy of the interstitial cells does not accompany degeneration of male germ cells, since the latter are absent from free-martin gonads. The interstitial cells of the free-martin gonad bear no relation to the sexual instincts, and little if any relation to the secondary sexual characters.

7. That the mammalian ovary possesses structures which have their morphological equivalents in the testis is demonstrated by the transformation of an ovary into a testis in the free-martin.

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Resumen por el autor, Carl Richard Moore,  
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Sobre las propiedades fisiologicas de las gonadas como determinantes de los caracteres somáticos y psíquicos.

### III. El hermafroditismo artificial en la rata.

La transplatación de una parte de la glándula sexual de una rata albina en otra rata del sexo opuesto, con una de sus glándulas sexuales normal, ha sido efectuada por el autor en ratas de 20 a 50 dias de edad. Las gonadas transplantadas crecieron en el animal de sexo opuesto en el cual fueron implantadas, y persistieron durante un periodo de más de ocho meses. Los cortes histológicos de los injertos, una vez que estos se implantaron, demostró que el ovario transplantado crece y persiste en una condición funcional en un macho con un testículo. El crecimiento y la diferenciación de los folículos de Graaf prosiguió de un modo normal hasta el periodo de la maduración, demostrando esto que el ovario persiste en estado funcional. Después del periodo de la maduración los folículos experimentan atresia en vez de ovulación y las masas foliculares se transforman en células intersticiales. Trozos de testículo transplantados en una hembra persisten durante un periodo de ocho meses, aunque aquella presente un ovario. El testículo transplantado no produce espermatozoides, porque los casos de transplatación, sin excepción en los mamíferos, van acompañados de la atrofia del epitelio germinativo. El autor describe la estructura de los injertos que prendieron, discutiendo su significación general. Los injertos de ovario permanecen en estado funcional en un macho con un testículo normal durante un periodo de ocho meses; esto demuestra que no existe aparente antagonismo glandular sexual en el caso de la rata.

# ON THE PHYSIOLOGICAL PROPERTIES OF THE GONADS AS CONTROLLERS OF SOMATIC AND PSYCHICAL CHARACTERISTICS

## III. ARTIFICIAL HERMAPHRODITISM IN RATS

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FIFTEEN FIGURES

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## I. INTRODUCTION

In view of the occasional conditions of hermaphroditism or pseudohermaphroditism, especially among mammals, wherein certain organs or tissues characteristic of both the male and the female occur in the same animal, and in view of the observations of Prof. F. R. Lillie on the development of the free-martin, and especially after the work of Steinach and others on the transplantation of gonads from one sex to the opposite one, there is a decided tendency to assume that one sex gland exerts a deleterious influence upon the other, perhaps through the mediation of an internal secretion from the gland. Various degrees of an inter-

sex condition are known not only for mammals in general, but also for the human individual, but in every such case on record there is an absence of function noted for one or for both sex glands, at least so far as production of germ cells is concerned. In a large majority of cases in which tissues or organs of both sexes are present, either separate or in a mixed condition, the animal is sterile; usually neither gland is capable of producing the sex cells that it normally produces.

Lillie's study of the free-martin<sup>1</sup> has revealed a powerful influence of the internal secretions of the developing gonad on the sexual apparatus of a developing foetus—so powerful, in fact, that the whole trend of zygotic determination of sex may be interfered with. And since the free-martin condition demonstrates that secretions are produced by the sex glands long before birth, we should be able to determine whether there is an antagonistic action between the secretions of the sex glands by transplanting gonads of the two opposite sexes into the same individual shortly after birth, or by transplanting a gonad into an animal of the opposite sex without castrating the latter. The success of such transplantations is apparently much greater if performed in the early life of the animal, and, furthermore, in the event of an early transplantation more opportunity would be afforded for an antagonism to assert itself; in the event of the existence of such an antagonistic action, probably abnormal changes would appear in the sex glands as growth and differentiation proceed, and thus we would have definite experimental evidence bearing upon the causes of hermaphroditism.

In a reinvestigation of the possibilities of the gonads as modifiers of somatic and psychical characteristics,<sup>2</sup> the writer has transplanted gonads in rats and guinea-pigs; the normal sex gland was removed and a gland of the opposite sex substituted for it. In such cases many, but not all, grafts obtained vascular connections and persisted. During the series of operations some cases of transplantation were carried out without previous removal of the normal gonad; ovaries were transplanted into

<sup>1</sup> Lillie, '17.

<sup>2</sup> Moore, '19



male animals in which one testis had remained undisturbed, or testis material was placed in the body of a female one of whose ovaries was yet intact. Since in some of these cases the transplant persisted, other operations followed. The results of such a series of operations are made known and discussed in the following pages of this paper.<sup>3</sup> It may be stated in advance that in the case of the white rat there is no evidence of an antagonism existing between the adult sex glands. Both sex glands may persist in a functional condition in the same animal for periods of five or eight months, and there is no apparent reason to place a time limit upon them for a continuance of this condition.

It is with great pleasure that I acknowledge my indebtedness to Professor Lillie not alone for provisions that have made possible an undertaking of this investigation, but also for his constant interest in the problem as the work has progressed.

## II. DISCUSSION OF LITERATURE

In the immense amount of existing literature on gonad transplantation in various groups of animals<sup>4</sup> there are relatively few accounts of attempts to transplant, either gonads of one sex to the opposite sex with the normal gonad in place, or to transplant both kinds of gonads into the same animal.

A controversy arose between Herlitzka and Schultz concerning priority in having transplanted gonads of one sex to the opposite sex. Schultz ('00) reported positive results in the transplantation of ovaries to male guinea-pigs. Though he does not state whether the males were normal or castrated animals, it is inferred that the testes had not been removed.

Schultz's results have been criticised severely by Herlitzka ('00). The latter author reports results obtained from transplantation of ovaries in forty operated cases on the guinea-pig. Generally, the persistence of ovarian transplants was poor. Herlitzka found that the germinal epithelium of the ovary re-

<sup>3</sup> Moore, '20.

<sup>4</sup> For complete bibliography of earlier literature on sex-gland transplantation, see Marshall and Jolly, '07, Castle and Phillips, '11.

mained intact if the peritoneal capsule surrounding the ovary is carried over with the transplant. The majority of Graafian follicles became degenerate very early, but in two instances normal follicles were found in the graft a month or little more after the transplantation was made. The ova degenerated before other parts of the ovary, and out of all cases examined but one ovum was found after a period of forty-two days: thirty-nine cases were negative in regard to the presence of normal ova in the grafts. Herlitzka attempts to account for this early degeneration of ova by assuming that the ovum is the most specialized part of the tissue and that the specialized tissue is the first to suffer in a strange environment. The author's general conclusions of antagonistic influences of sex glands are that the presence of the testis does not impede or interfere with the vitality of ovarian tissue. However, this investigator was not able to obtain good persistence of tissue even in autoplasmic grafts (removal of ovary to another position within the same animal); hence his results, little better than negative evidence, shed little light upon a possible antagonism. He is of the opinion that Schultz utilized young animals for his experiments, for Herlitzka's impressions are that adult ovaries will not persist after transplantation.

Schultz ('00) reports five positive cases of ovaries transplanted into males with the transplanted ovary retaining its characteristics in quite a normal condition. He mentions no negative cases and neither does he state whether the animals were young or adult. Possibly they were young animals, and Foa ('00) has shown that there is great possibility of persistence if the transplants are made at an early date.

Marshall and Jolly ('08) transplanted ovaries of rats from one position to another in the same animal, as well as from one rat to another. Considerable success attended their efforts, as grafts after five to eight months showed the presence of follicles and corpora lutea. One attempt was made to graft an ovary into a male rat (presumably with testis present), according to them, "with some success. . . . The graft showed recognisable ovarian tissue in parts, but had undergone very con-

siderable degeneration." They conclude as to the physiology of the ovary: "The maintainance of the histological characters in successful grafts points to the retention by them of function." "The appearance of the uterus was found to bear a relation to the microscopic structure of the graft. If the latter had retained unaltered, or with little alteration, the typical characters of ovarian tissue, the uterus was found undegenerated" (p. 597).

Basso ('05), using guinea-pigs and dogs, attempted transplantation of ovaries to the male. And since results obtained from this procedure were no less successful than from those grafted into females, the author concluded that the presence of the testis did not prevent the persistence of the ovary. His results on the whole, however, were not especially good.

Steinach ('12 and later) maintains that a sex-gland graft will not grow in the animal that possesses a gland of the opposite sex. In young rats and guinea-pigs glands of one sex when transplanted into an animal of the same sex, or into an animal of the opposite sex that has been castrated, will persist and grow. And, in the latter case, Steinach maintains that as the animal develops it will assume the somatic and psychical characteristics of the sex represented by the graft. If, however, a gonad of either sex is grafted into an animal of the opposite sex with intact gonads, then the graft will fail to become vascularized, gradually shrinks, and very early disappears. Steinach assumes that a secretion from the interstitial cells of the one sex gland (a hormone) exerts a deleterious influence upon the opposite sex gland, thus preventing its growth. Later, however, Steinach considers that he was able partially to overcome this hormone antagonism by grafting simultaneously the two opposite sex glands into the same infantile, castrated animal; he was able to obtain a certain persistence of the two glands for short periods of time. He repeatedly denies any growth of an ovary grafted into a young male animal with intact testes, or growth of a testis grafted into a female animal possessing ovaries.

Sand ('19) also maintains that a gonad will not grow subcutaneously in an animal whose sex glands are intact, provided that the graft is from an animal of the opposite sex from the



host. He, too, was able to obtain growth of both kinds of sex glands if they were simultaneously grafted into a previously castrated animal. Sand was also able to graft an ovary into the substance of the testicle and have it persist in quite a normal state. He does not agree with Steinach's idea of a sex-gland antagonism, but supposes some kind of 'atreptical immunity' of the non-castrated organism.

In birds Hanau ('97) observed that a testis, transplanted into hens possessing ovarian tissue, became encapsulated, necrotic, and was absorbed, though if grafted into cocks it persisted with living spermatozoa.

The free-martin starts its development as a female (Lillie, '17), but, supposedly due to the influence of an internal secretion of the developing testicle (a hormone), the ovarian development does not proceed in its normal fashion, but is partially or completely suppressed. Not alone does the indifferent gonad fail to develop into an ovary, but it assumes many of the characteristics of a developing testicle. The end result is that the 'determined' female genital system assumes during its development, and in the adult of the free-martin, many characteristics of that in a male individual.

### III. MATERIAL AND METHODS

The common white rat (*Mus norvegicus albinus*) has afforded excellent material for the study of the possibilities of sex-gland antagonism. Previous experiments have demonstrated the ease with which transplantation may be effected,<sup>5</sup> especially when the host of the graft had previously been deprived of its normal gonads. In this paper, however, we shall only consider the fourteen positive cases of persistence of sex-gland tissue grafted into an animal of the opposite sex with one of its normal gonads undisturbed. The grafts have persisted for periods of  $4\frac{1}{2}$ , 5, and  $7\frac{1}{2}$  months, and number twenty-eight grafts, over twenty of which have been sectioned, stained, and studied microscopically (table 1).

<sup>5</sup> Steinach, '10, '11, '12, and Moore, '19.

The transplantations were made on young animals twenty-six to fifty-seven days after birth. The procedure was usually as follows:

A male and a female were etherized at the same time, the coelomic cavity of both exposed, and one sex gland of each removed, the second gland remaining undisturbed; the extirpated gland furnished the material for the transplant to the rat of

TABLE 1

SERIAL NUMBER	AGE OF ANIMAL AT GRAFTING	AGE OF GRAFT WHEN ANIMAL WAS KILLED	NUMBER OF GRAFTS PERSISTING
<i>Males with ovarian graft</i>			
	<i>days</i>	<i>days</i>	
40-4 A2B2II	33	152	3
40-4 A2B2IV	33	152	2
49 A	26	232	2
49 C	28	230	2
51 D	48	130	2
51 E	48	130	2
51 G	48	130	2
51 H	48	130	2
<i>Females with testis graft</i>			
40-4 A2B2III	33	152	2
40-4 A2B2V	33	258	2 <sup>1</sup>
49 B	57	232	2
51 A	48	130	1 <sup>1</sup>
51 B	48	130	2
51 C	48	130	2

<sup>1</sup> Not sectioned.

opposite sex. An ovary from the female was cut into two pieces, these being placed subcutaneously, intramuscularly, or intraperitoneally in the male; and in like manner the testis material, cut into small pieces, provided the graft material to be placed in the female. Slight injury to the tissue at the site of transplantation produced a local hyperemia and with the small-sized pieces of the graft more chance was provided for the establishment of vascular connections. The animals were kept in an incubator for from twenty-four to thirty-six hours after

recovering from the anaesthetic. The rats were so marked and caged that individual animals could be recognized at all times. At the desired time the animals were killed, the grafts removed from their positions, as was also the normal intact gonad, and the tissue preserved in Bouin's fluid. Paraffin sections were prepared and stained with Delafield's hematoxylin and eosin.

There were a considerable number of cases in which the grafts did not persist, as well as a small number of deaths of animals before a study of the glands could be made; however, positive persistence of grafts was obtained in over 50 per cent of the cases.

Operations of the same character as those described above have been carried out on guinea-pigs instead of rats; these have not been of any considerable number, and all results have so far been negative. Experience has shown, however, that grafts grow more readily in rats than in guinea-pigs, and no especial significance has been attached to the non-persistence in cases where a sex gland of the opposite kind from the graft was intact in the host.

Due to the fact that many pieces of tissue of large size were taken from the rats at the time of killing and that one is not certain of the character of the tissue so taken until after sectioning and staining, all tissue was preserved in the same way, cut at the same thickness, and stained with the same stains. So far the writer has made no attempt to stain differentially the interstitial cells of these grafts; many times the material is decidedly unfavorable for such staining, as the tissue, especially the testis, may be considerably infiltrated with connective tissue which had invaded it during growth. The interstitial cells of the rat testis are, moreover, relatively small, and possess little specifically characteristic. The present paper will deal with considerations of the gland tissue in general rather than with the interstitial cells as specific tissue.



## IV. GENERAL HISTOLOGICAL CONSIDERATIONS

*A. The ovary*

Before proceeding to a detailed consideration of the individual grafts (section V), it will be necessary to mention a few general features of the histology of the grafts in order that the writer's view-point may not be misunderstood.

The subcutaneous grafts were usually quite prominent after the skin of the animal had been removed, and in many cases they could be distinctly palpated before death. Those placed in the intraperitoneal position were usually visible when this cavity had been opened, for they ordinarily project slightly into the cavity, being surrounded and encapsulated by connective tissue. Differences in the types of implantation are associated with the site of the original graft and the condition of the tissue when the graft was made. Figure 1 (a section of a five-month graft from animal 40-4A2B2 IV) indicates one type of implantation. This was a subcutaneous graft resting upon the external oblique muscle, and at the time of transplantation the small peritoneal sac surrounding the ovary was carried along with this part of the ovary in such a way that it has been retained. In such cases of retention the cortex of the ovary is protected from other tissues surrounding it and it remains in a typical condition, but if the cortex comes in contact with surrounding tissue the substance of the ovary grades almost imperceptibly into the encapsulating tissues and does not retain the definite covering of the ovary. A section of the oviduct accompanies this graft.

There has been no attempt made to determine the amount of growth in any separate graft, for at the time of operation the graft material was prepared by cutting the young ovary into two pieces with scissors, and these pieces were many times of unequal size at the beginning. Furthermore, one of the pieces may consist of but little else than oviduct,<sup>6</sup> while the other consists principally of the gland itself, and in view of this fact some of the grafts recovered from the sites of implantation show

<sup>6</sup> No attempt was made to remove the oviduct, fimbria, or peritoneal covering of the ovary at the time of transplantation.

little else than sections of the oviduct, while others consist almost entirely of ovary tissue. That growth has followed implantation, made possible by the very good vascularity established, is indicated not only by the presence of numerous follicles

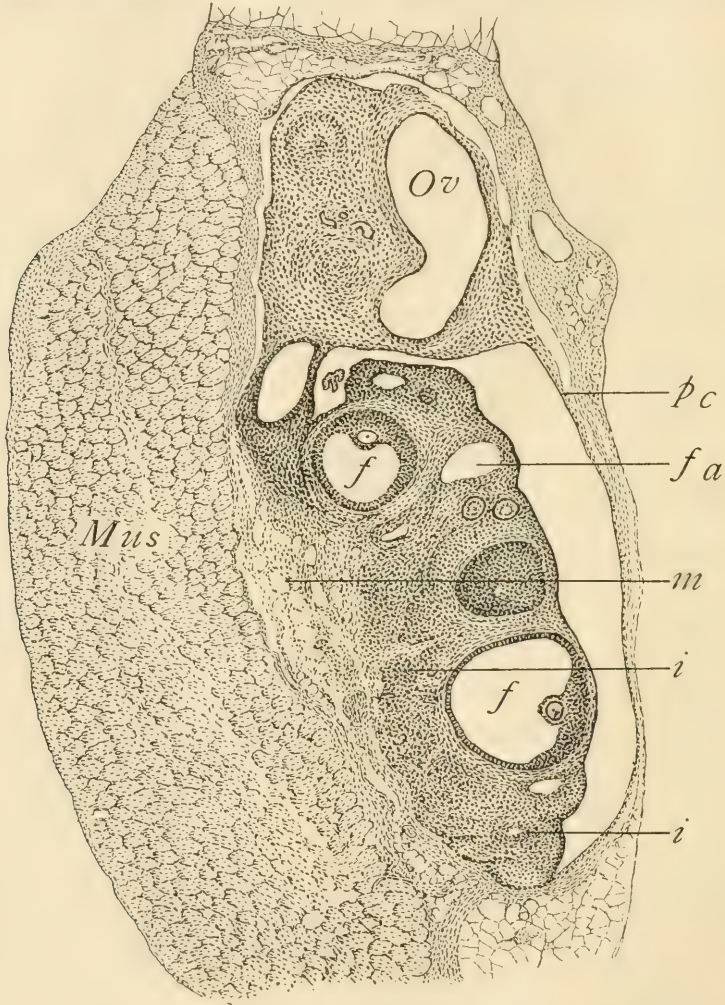


Fig. 1 Section of subcutaneous ovarian graft recovered 152 days after grafting into male rat 40-4 A2B2 IV (one testicle present). *f*, follicles near stage of maturity; *fa*, atretic follicle; *i*, interstitial cell mass; *m*, medullary region; *Mus*, external oblique muscle; *Ov*, oviduct; *pc*, peritoneal capsule.

of various sizes within the graft, but also by the fact that the cells of the follicles show numerous mitotic figures. This, as well as the very evident changes of other kinds, clearly indicates that the sections of tissue do not merely represent the type of structures present within the tissue at the time of transplantation, but show that there have been changes going on in the ovarian tissue during its residence within the male for several months, and that these changes are for a large part, essentially similar to those changes that would have ensued had the gland remained undisturbed. How long the ovary might have remained in this abnormal environment is of course unknown, but for the eight and one-half months' time it remained in the male animal there is little evidence of degeneration of the tissue as a whole or of resorption of the graft.

Histologically, the substance of the grafts consists of ovarian tissue within which are preserved all stages of the Graafian follicles. In the majority of the grafts primordial follicles (a primordial ovocyte surrounded by a single layer of follicular cells) are relatively abundant, but in some the younger follicles, though present, are not the predominating feature. Figures 3, 4, 5, and 6 represent a few of the progressive stages in the development of a follicle, and all except figure 6 are from an ovarian graft taken from the male (49 A) 230 days after the original operation. Each of the stages and many others are represented not only by this graft, but also by practically every graft in which the ovarian tissue is retained, and it does not allow of the least doubt that the production of mature follicles proceeds under these abnormal conditions. In figure 6 the ovum, enclosed by its discus proligerus, has just extruded a polar body in the maturation stage; it is supposed that this is the first polar body. Beyond the maturation stage, however, the further normal activity of the follicle is interrupted, and instead of ovulation and corpus luteum formation, the follicles undergo atresia.

In the normal female the ovum forms its second maturation spindle while in the follicle and remains in this condition until ovulation, but it is well known that those ova which are retained by accident within the follicular cavity at the time of ovulation,



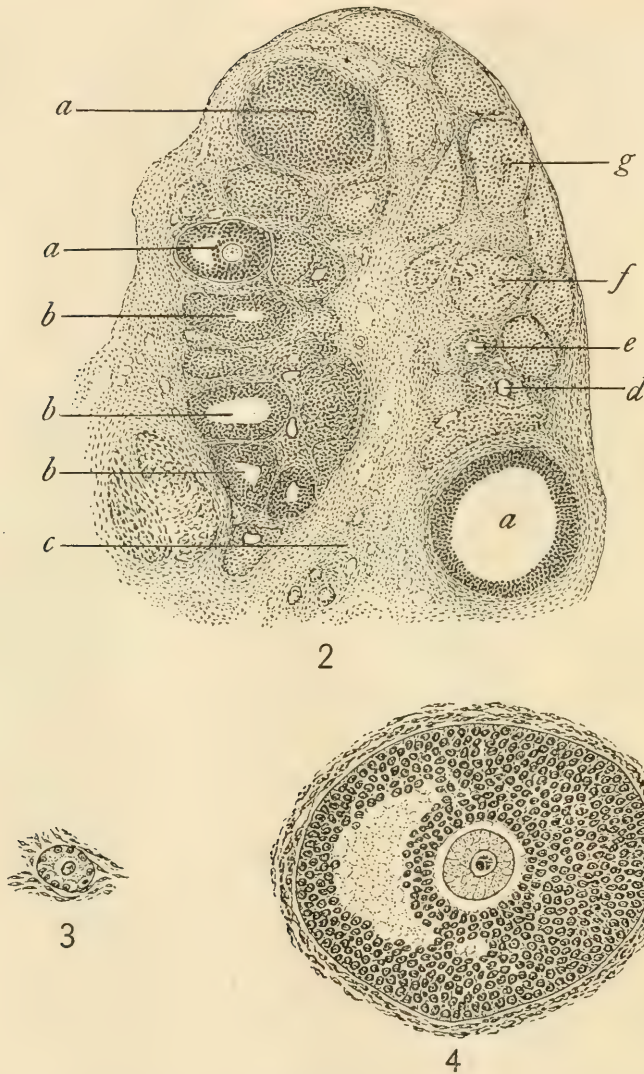
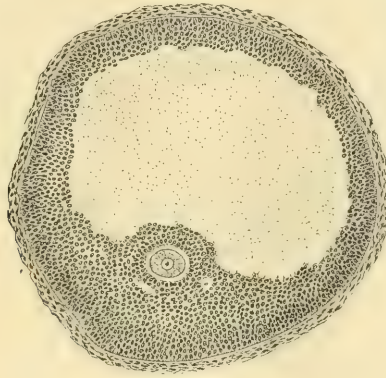


Fig. 2 Part of a section from a 130-day ovarian graft from male 51 E, showing relative number of normal and atretic follicles. a, normal follicles; b, early atretic follicles with large follicular cavities; c, medullary portion of ovary; d, e, f, g, follicles late in atresia showing last traces of follicular cavities.

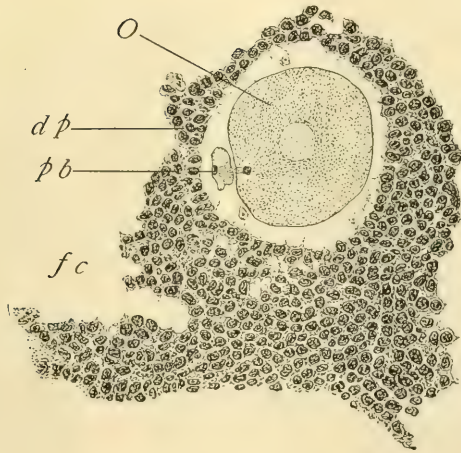
Fig. 3 Primordial follicle in a 232-day ovarian graft from male 49 A.

Fig. 4 Follicle with beginning follicular cavity (from same graft as fig. 3).

as well as those follicles near the stage of maturation when corpora lutea are formed, do not remain in this stage, but undergo atretic changes. Also before the onset of sexual maturity (in



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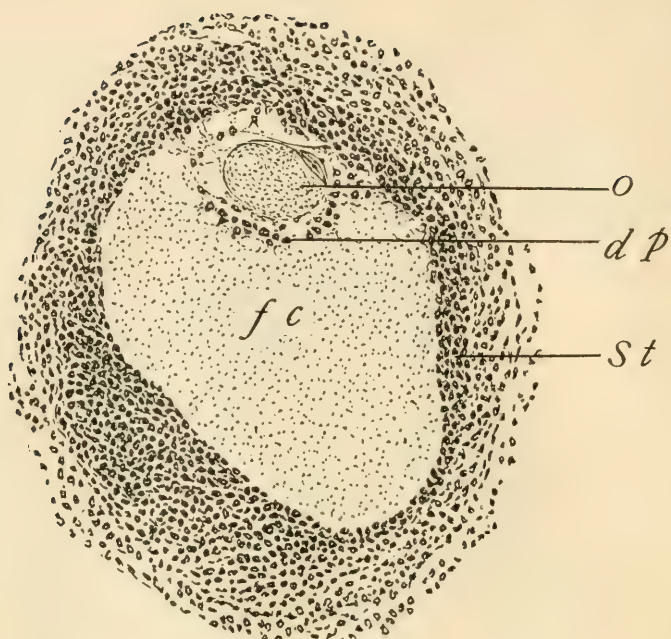


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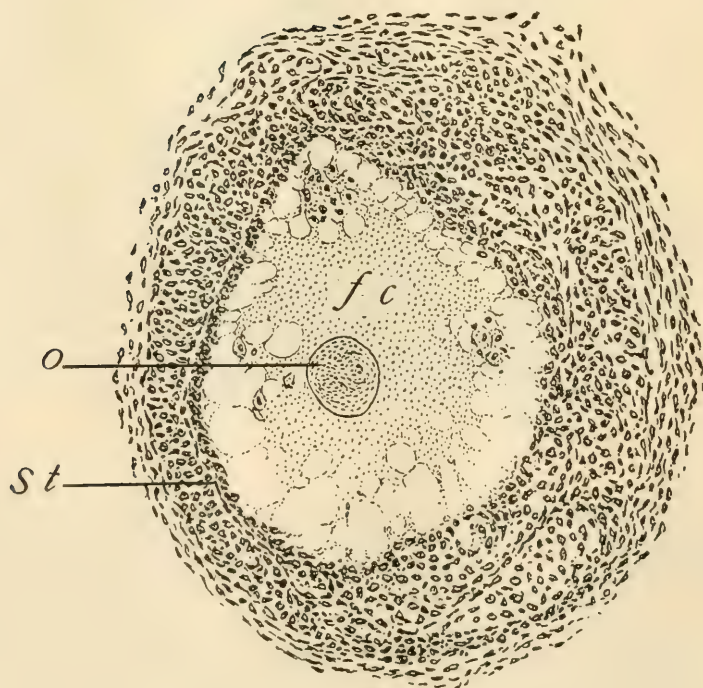
Fig. 5 Follicle about the stage of maturity (from same graft as figs. 3 and 4).

Fig. 6 Portion of mature follicle with ovum and polar body (from 230-day ovarian graft of male 49 C). The ovum is surrounded by the degenerating discus proligerus. *dp*, discus proligerus; *fc*, follicular cavity; *O*, ovum; *pb*, polar body.

the rat) the follicles proceed in their development to about the stage of maturation—i. e., the follicular cavity is well developed and the ovum is surrounded by a distinct discus proligerus



7



8



—and instead of this stage being followed by ovulation the follicles undergo atresia. Figure 12 is a section of a normal rat ovary thirty-six days after birth, and within this one section four atretic follicles (A, B, C, D,) showing a section of the ovum represent quite well the conditions encountered here; in follicle A the ovum has undergone parthenogenetic segmentation, three of the four cells being visible.<sup>7</sup> There is, then, a similarity between the ovary before the onset of sexual maturity and the ovarian graft in a male animal, inasmuch as both conditions result in the destruction of the follicles and contained ova instead of the normal processes of ovulation and extrusion of the ovum from the follicles. Figure 7 shows a follicle from a 232-day graft (male 49 A) in an early stage of its atresia. Here the discus proligerus is undergoing dissolution and the ovum itself is seen to have assumed an elliptical shape instead of the usual rounded condition, and it contains a mitotic figure bearing perfectly definite chromosomes arranged equatorially; it seems possible that this may be the second maturation spindle, at which stage ovulation should intervene, though of this there is no absolute certainty, since the first polar body was not observed. This condition is encountered quite frequently in these grafts, and some twenty-five or thirty ova showing definite spindle and chromosomes have been studied. Figure 8 (from the same graft as fig. 7) is a follicle further advanced in its atresia. Here the discus proligerus has entirely disappeared, excepting possibly the loose scattered cells within the follicular cavity, and the ovum, one piece of which is visible in the section, has undergone fragmentation; in this figure, one can see that the stratum granulosum is intact and is not undergoing dissolution. It should be emphasized that not one, but scores of cases of fragmentation of

Fig. 7 Atretic follicle showing ovum in spindle stage (from same graft as figs. 3, 4, and 5). *fc*, follicular cavity; *o*, ovum with spindle; *St*, stratum granulosum.

Fig. 8 Follicle showing more pronounced atresia (from same graft as figs. 3, 4, 5, and 7). *fc*, follicular cavity; *O*, one fragment of degenerating ovum; *St*, stratum granulosum.

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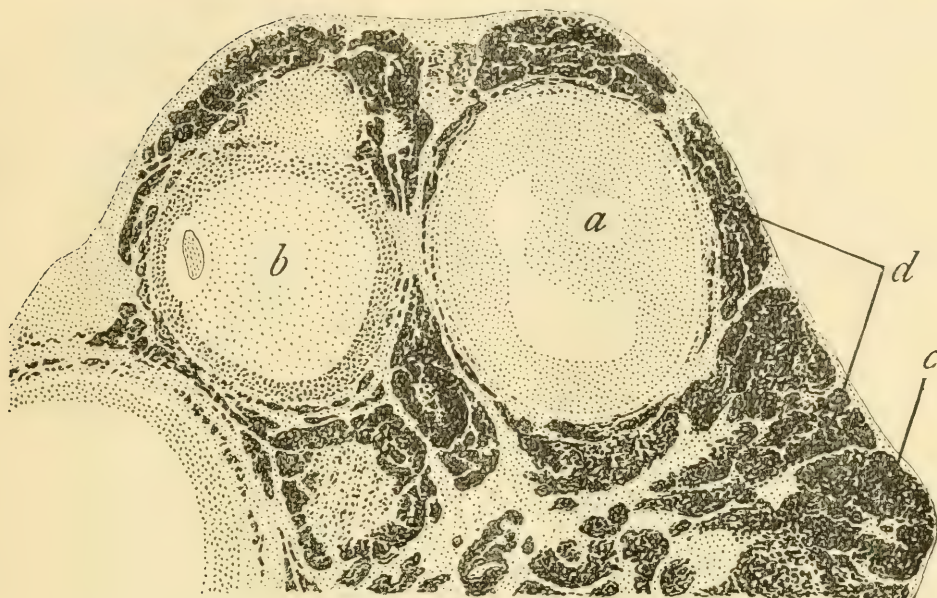
<sup>7</sup> The nuclei are not well shown in the figure, but they are visible in the preparation.

ova have been studied and the steps in this degeneration form a perfectly distinct graded series in its atypical progression. Figure 2 (a section of a four and a half months' graft from male 51 E) indicates very well the relative number of atretic and normal follicles present in one section of some of these grafts; follicles a and b are normal.

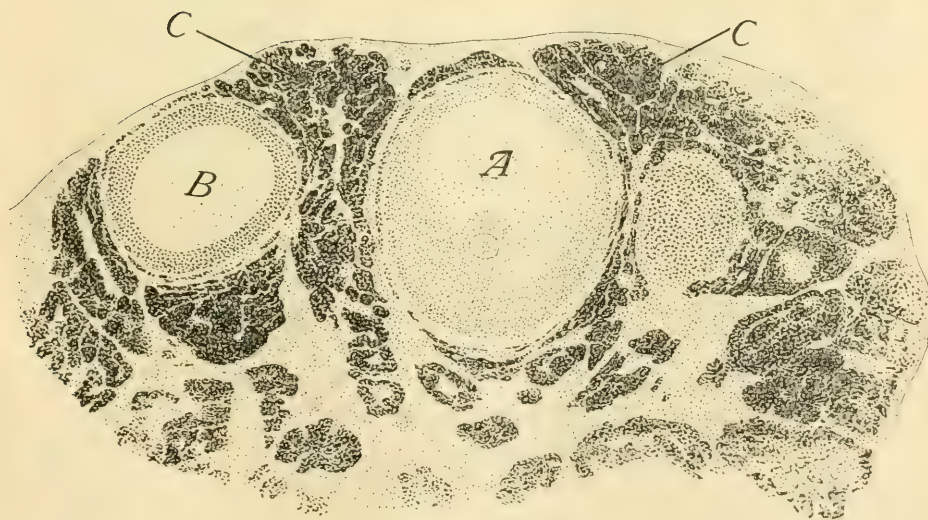
The normal ovary, however, consists not alone of follicles (stratum granulosum, ovum, internal and external thecal layers), but also situated between the follicles and in the midst of the connective-tissue stroma are cells larger than connective-tissue cells, possessing well-stained nuclei and a reticular-like cytoplasm in ordinary stained preparations. Fixed with a solution containing osmic acid, however, these cells—singly or in groups—are very distinct; they reduce osmic acid and, if mounted unstained after such a fixation, they stand out as masses of blackened cells in contrast to the follicles and connective tissue of the ovary, both of which are but faintly seen. Such cells are usually spoken of as 'lipoid containing cells' and they constitute what are ordinarily called interstitial cells.

The question of the interstitial cells of the ovary is one concerning which few definite conclusions can be drawn. The literature is an enormous one and in so far as the theoretical considerations are beyond the scope of this paper a review of this literature will not be attempted.<sup>8</sup> But in so far as reference must be made to the interstitial cells of these grafts, the writer wishes to define his position in reference to this matter. The term 'interstitial cells' will be applied, in the case of the ovary, to those cells of relatively large size, situated between the follicles, in the connective-tissue stroma, whose nuclei are well stained, cytoplasm of reticular character, and which blacken after fixation with osmic-acid preparations. Figures 9 and 10, small sections of a normal rat ovary thirty-six days after birth (fixed in strong Flemming's solution and mounted unstained), portray the characteristic masses of these cells; the lipoid-laden cells of the theca interna are also plainly visible. And at least one origin

<sup>8</sup> For a review of the literature on interstitial cells the reader is referred to the papers of Kingsbury and Rasmussen.



9



10

Fig. 9 Part of a section of a young normal ovary (sixty days after birth) fixed in osmic-acid preparation. a, normal follicle; b, follicle beginning atresia (small part of ovum present); C, follicle late in atresia reverting to interstitial cells; D, interstitial cells.

Fig. 10 Part of another section of same ovary as figure 9. A, normal follicle showing ovum; B, follicle in early atresia; C, interstitial cells.



of such cells is so clearly demonstrated in these grafts under consideration that there is no hesitancy in making mention of it while considering the fate of the atretic follicles with which these grafts abound.

The history of the follicle has been described in a very general way up to the stage at which the ovum fragments and the products of the destruction lie free within the follicular cavity. In every graft there are numerous follicles in which all but the debris of the ovum has disappeared, and figures 8 and 11 show clearly that the stratum granulosum of such follicles remains intact and unbroken. But these cells of the stratum granulosum have undergone considerable change since they were cells of a normal follicle (not well shown in the drawing), for the cells are larger and the cytoplasm has taken on a pronounced reticulated appearance. These changes are still more pronounced as atresia is advanced, and if the follicular cavity is noticed one sees that it becomes smaller and smaller as the changes go on, until only the least vestige of a cavity is discernible; the stratum granulosum cells remain in their characteristic position and the entire follicular mass is surrounded by a connective-tissue layer that is especially well demonstrated after staining with Mallory's triple stain. Figure 11 (a section of an eight-month graft from male 49 A, stained with hematoxylin and eosin) shows at B a follicle in which the cavity has almost entirely disappeared, but the large mass of granulosa cells are clearly surrounded by its connective-tissue layer (probably the external theca). Follicle A is one in an earlier stage of atresia, while the tissue at C is the remains of follicles whose degeneration has taken them still farther; from this figure the gradual progression of atresia is shown in these three steps, from the definite structure in A through the condition exhibited by B and finally the remains of such a destruction at C. Reference again to figure 2 shows the follicles d, e, f, and g with the small-sized follicular cavities, but others in the same section have lost all visible traces of the cavity and the masses of cells are distinctly or indistinctly surrounded by the connective-tissue layers, depending upon how far the changes have progressed. It is to such masses of cells that the



Fig. 11 Portion of 232-day ovarian graft (same graft as figs. 3, 4, 5, 7, and 8), showing atretic follicles and interstitial cells. A, atretic follicle (no trace of ovum remains); B, follicle in more advanced atresia with slight remnant of follicular cavity (fc); C, interstitial cell masses. fc, follicular cavity; St, stratum granulosum.

writer applies the term 'interstitial cell,' and it is such cells of the ovary that are at least involved in the reduction of osmic-acid preparations.

Since the graft material was all preserved in Bouin's fluid, recourse has been had to very young normal ovaries for a demonstration of the property of reducing osmic acid by the interstitial cells. Histologically and developmentally, such interstitial cells of a young ovary are identical with the same type of cell in the grafts, and inasmuch as atretic follicles are abundant in both instances it will throw some light on the changes taking place in the grafts to examine the young ovary. Reference to figure 12 will remind one of the atresia that is going on in such an immature ovary and the relative number of these follicles to normal ones. Moreover, it will show that the stratum granulosa of the follicles remains intact during the atretic changes, for in these abnormal follicles the discus proligerus is undergoing dissolution while the granulosa cells are present, but also undergoing the characteristic changes of the cells as they acquire their lipid content. In the osmic preparations the gradual steps in these changes are well represented. Figures 9 and 10 have for comparison in each case a normal follicle and one beginning atresia. Follicle A is the normal follicle and the granulosa cells possess but little osmic-blackened material, while follicle B in each case has begun to degenerate (in fig. 9, B, a small fragment of the contained ovum is visible in the section). In comparing follicle A with follicle B in each figure one can readily see that the blackened material in the granulosa cells is much more apparent in the degenerating follicle, and (using other follicles) as this atresia progresses the intracellular lipid material is still more pronounced, until finally such masses of cells as are shown at C (fig. 9) represent the original follicle; these masses are situated between the follicles, some may contain a very small representative of the original follicular cavity, and in this characteristic position, and behaving as they do after osmic fixation they are spoken of as the interstitial cells of the ovary.

This origin of this type of cell can be followed in practically every graft of the series, and with but one possible exception the



general behavior of the follicles seems to follow this line of development; this one exception is a case to be described in the next section, where there are cells that resemble to a very great degree



Fig. 12 Section of normal ovary from rat thirty-six days after birth, showing normal and atretic follicles. Follicles A, B, C, and D show beginning atresia (the discus proligerus is degenerating). Ovum in follicle D shows parthenogenetic cleavage, three of the four cells visible.

the ordinary lutein cell of the corpus luteum. Aside from this one case, there has been absolutely no indication that ovulation has occurred in any of these grafts.

The end result of follicular atresia is the conversion of the follicular masses (stratum granulosum) into the large-sized cell masses, blackening with osmic acid, which are commonly spoken of as the interstitial cells of the ovary.

### *B. The testis*

Transplantation of testis has been far less successful than the ovarian transplantations, as was found true in the writer's earlier experiments. However, practically all experimental work has indicated that the testicle is a more labile organ than the ovary and more subject to modification than the latter. So far as the writer is aware, all cases of testicular transplantation in mammals have resulted in an interference with spermatogenesis; the spermatocytes and spermatozoa disappear and usually only a few scattered cells remain in the tubules, such cells being considered as the cells of Sertoli. Many investigators, however, have found that mere ligation of the vas deferens results in a similar disappearance of the germinal epithelium, so that it is only to be expected that the tubules of persistent grafts would be devoid of their normal germinal epithelium.

At the time of the transplantation a small part of the testis was introduced into a subcutaneous pocket in the female, and as the substance of the organ readily escapes from its connective-tissue capsule the loose tubular mass was separated by the ingrowth of connective tissue into the implanted material during the process of wound healing. When the graft was sectioned the tubular mass was found closely encapsulated and infiltrated with connective tissue, as can be seen from figure 13 (section of a 152-day testicle graft from female 40 4 A2B2 III). The tubules are perfectly distinct, well rounded in section, even though containing no cellular material other than the Sertoli cells, while connective tissue surrounds the entire mass and is present between the tubules. Figure 14(a section of a 232-day graft from female 49 B) is a more highly magnified drawing of a few tubules.

The principal tissue of the testicle, aside from the seminiferous tubules, is the interstitial cell masses normally located between

the adjacent tubules and under the tunica albuginea (fig. 15 for the normal relations). So long as the testis is an encapsulated organ, these cells are recognized from their location, but as soon as the tubules are separated by ingrowth of connective tissue as occurs in these grafts, it becomes more difficult to recognize

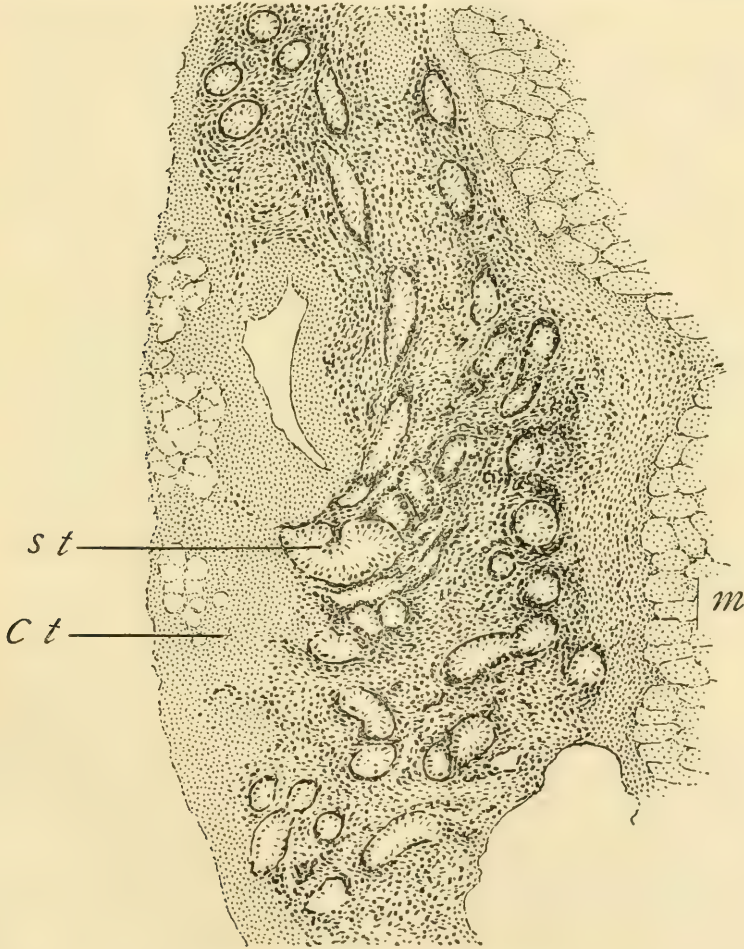


Fig. 13 Portion of a subcutaneous testicle graft 152 days old (from female 40-4 A2B2 III—one ovary present), showing seminiferous tubules scattered in connective tissue. *ct*, connective tissue; *m*, external oblique muscle; *st*, seminiferous tubules.



the cells as specifically interstitial cells. The masses of interstitial tissue in the transplanted gland, as seen in figure 14, contain cells that are foreign to the same location in the normal condition. Two types of cells especially are found in practically every testis graft sectioned. One type is a comparatively large

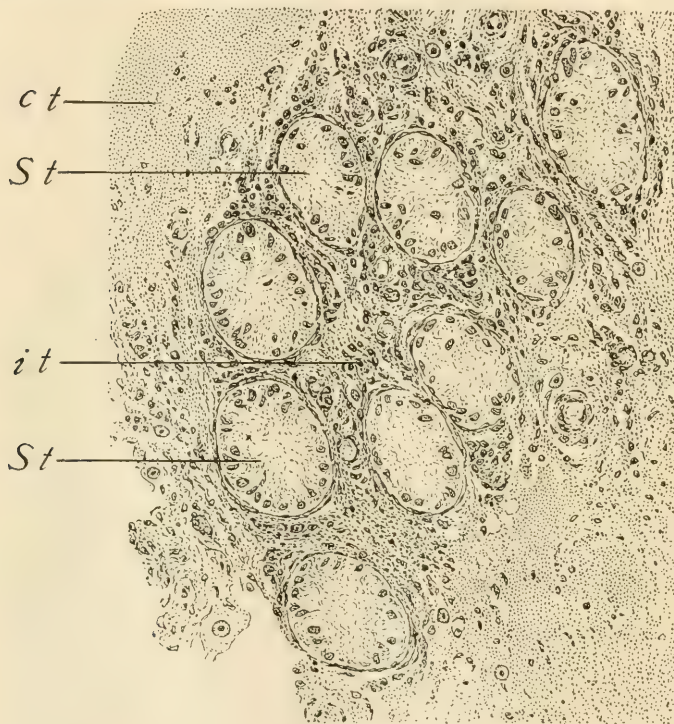


Fig. 14 More highly magnified portion of a 232-day testicle graft from female 49 B. *ct*, connective tissue; *it*, interstitial cells; *St*, seminiferous tubules containing sertoli cells.

cell, chiefly distinguished by the large eosin-stained granules within the cytoplasm, the nucleus being usually nodular in form or composed of distinctly separate parts; these cells are regarded as the typical eosinophile cell of the vascular system, but their significance in this locality and in such numbers is entirely unknown. Usually they are somewhat indicative of a more or less

chronic state of inflammation, and they have not been found associated with the ovarian grafts. The second type of cell is about the size of the eosinophiles, possessing homogeneous cytoplasm and distinct well-rounded and well-stained nuclei. Depending upon the extraction of the stain from the preparations,

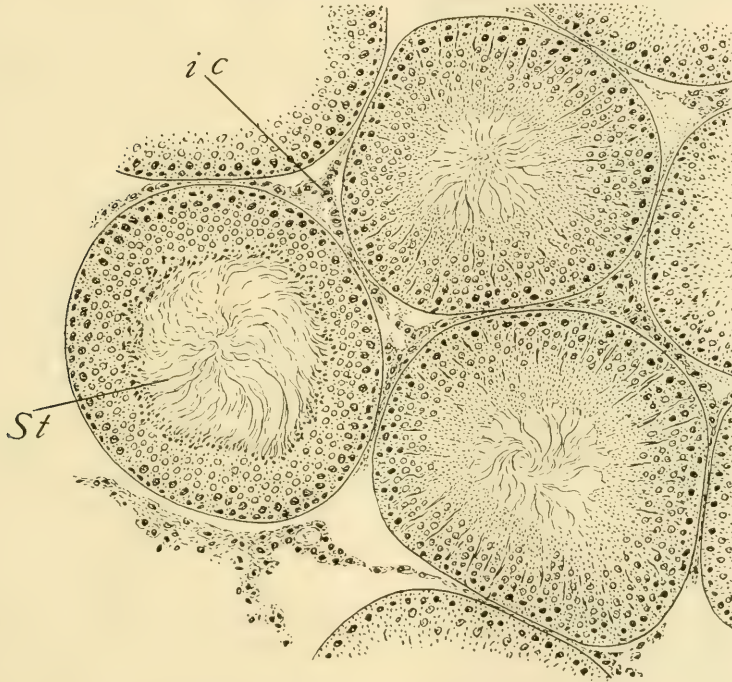


Fig. 15 Tubules of normal testis of rat 49 A (one testicle was removed at operation, and the rat had borne two large subcutaneous ovarian grafts for 232 days—graft 49 A, 1 and 2). *St*, seminiferous tubules containing spermatozoa; *ic*, interstitial cells.

the cytoplasm is light blue in color or a slight pink. These cells are entirely foreign to the normal testis and their significance is not understood. It is difficult to decide whether the quantity of interstitial cells is increased or decreased, for the interstitial cells of the rat's testis have no distinctive features that will class them unmistakably as interstitial cells. However, there

is not an especially marked increase in the quantity of interstitial cells as has been described by some writers. It is true that the tubules are quite widely separated and the intertubular spaces are occupied by cells, but there has been a very considerable invasion of connective tissue as well as an abundance of the cells to which particular attention was directed.

#### V. SPECIFIC CONSIDERATIONS OF INDIVIDUAL GRAFTS

##### A. *The ovary*

Included in this section are the more specific details of the histology of the individual grafts of ovaries recovered from the male animals into which the transplantation had been made at an earlier date.

*Animal 40-4 A2B2 II (table 1).* Male rat, born Nov. 14, '17. Dec. 17, the left testicle was removed, and two small pieces of ovary from a sister were grafted subcutaneously. May 18, '18, animal killed; left testis is normal, sperm sacs are normal; one ovarian graft quite prominent, the second one small; the largest graft was sectioned for study.

*Microscopic.* The principal part of the persisting graft is made up of sections of the oviduct which was carried along with the piece of ovary at the time of transplantation; the ovarian tissue proper is very small in amount, since it is present in only about fifty sections ( $10\mu$  thick). The ovarian tissue proper is so small in amount that in some sections the one large normal Graafian follicle appears not to be surrounded by ovarian stroma. However, close examination shows a small amount of encapsulating stroma tissue, and to one side of the large follicle are a number of atretic follicles of very small size. There is little of especial interest in the small abnormal follicles, but there is considerable interest associated with the apparently normal follicle evidently very close to the stage of maturation. The stratum granulosum is normal in appearance, the thecal layers of the follicle are normal; there is a large follicular cavity and the ovum appears absolutely normal and possesses a well-marked normally staining nucleus; the ovum is surrounded by the discus proligerus in an entirely normal manner, and in short, the follicle



is apparently perfectly normal in spite of the fact that so little ovarian tissue is present that in places scarcely any stroma tissue is apparent at the peripheral margins of the follicle.

In this graft there has been apparently a large amount of resorption and in the sections studied there are abundant evidences of inflammatory changes having taken place. The cavity of the fallopian tube shows masses of leucocytes throughout the entire 200 sections (the ovarian tissue proper is present in only about fifty sections) and within the lymph spaces of the oviduct and areolar tissue surrounding the ovarian tissue there is a great amount of lymphocyte infiltration. Evidently, associated with the inflammation and the small amount of stromal tissue, there has been a considerable amount of destructive changes, possibly an infection of the graft, that has resulted in a degeneration of part of the graft; but the remarkable thing is that there can be such a normal, well-developed follicle at such a late stage of formation in the small amount of ovarian tissue that has persisted throughout the entire 152 days of residence of the graft in the male animal. This indicates a comparatively great resistance on the part of the follicle and contents rather than an extremely non-resistant condition, as held by Foa. There are no evidences of lutein tissue or of ovulation in this graft.

*Animal 40-4 A2B2 IV.* Male rat, born Nov. 14, '18. Dec. 18, left testis removed, two small pieces of ovary from sister grafted subcutaneously. May 18, '19, animal killed; right testis is normal, sperm sacs normal, both grafts had persisted; one was sectioned.

*Microscopic findings.* The ovarian graft recovered 152 days after implantation in the male rat, one of whose testes had remained in position, is of considerable size and perfectly normal histologically. Figure 1 shows a section of the graft which is surrounded by a muscular and fibrous capsule. The original peritoneal sac covering the ovum has been retained and provides a sac-like covering for the graft in such a way that the cortex does not come in contact with other tissues. Both medulla and cortex are distinct, including the very large number of follicles of all stages of development situated in the latter. A section of

the oviduct accompanies the graft, having been carried along with the ovary at the time of transplantation.

The graft proper, cut  $10\mu$  in thickness, consists of 360 sections. Graafian follicles can be seen in any section of the gland, and these represent all stages of development from the primordial to the mature follicle. The younger ones consist of a very small ovum surrounded by a single layer of follicular cells; older ones are surrounded by a greater number of cells and may show just the beginning of formation of a cavity, while still others contain a follicular cavity of larger size; still older follicles show a very large follicular cavity containing the ovum surrounded by the discus proligerus, the whole follicle being in a stage of, or very near, maturity. All gradations of atretic follicles are likewise present from stages showing the beginning fragmentation of the ovum, through larger atretic follicles containing no evidence of an ovum, to small atretic ones, in some of which only the suggestion of the remains of the old follicular cavity can be made out. There is such a close gradation that many times it is difficult to say whether the region represents an atretic follicle or simply the large reticular, interstitial cells of the stroma.

In this graft seventy normal follicles have been counted; these represent all stages of development and all contain the ovum possessing a distinct and well-stained nucleus. The atretic follicles are so numerous and many times it is so difficult to call the structures follicular masses or interstitial cells, that no attempt has been made to enumerate them. There is an abundance of very young follicles situated just within the periphery of the graft, many of them containing normal ova; others, however, perfectly distinct as follicles, show absolutely no traces of an ovum. Within the granulosa cells of the larger follicles a great number of karyokenetic figures indicate that the follicles are growing in a perfectly normal fashion and dispel the idea that they are only persisting follicles at the same stage as when the graft was made.

There is considerable evidence of new follicle formation about the periphery of the graft for certain regions show the presence of perfectly evident follicles, with or without an ovum, but the

indication is not definite proof that such a process is going on; no germ cells aside from those included within a follicle have come to my attention.

There is no trace of a corpus luteum in any of the sections of the graft nor is there any evidence that ovulation has ever taken place.

*Animal 49 A.* Male rat, born Nov. 6, '17. Dec. 1, the right testis was removed (used to graft into a female) and two pieces of ovary were grafted subcutaneously into abdominal fascia and muscle. Aug. 22, '18, animal killed; both ovarian grafts had persisted, the left testis was normal, sperm sacs were normal. Both ovarian grafts sectioned.

*Microscopic observations.* Graft no. 1. After its residence in the male host, possessing one testicle, for a period of 232 days after transplantation, the ovarian graft shows characteristic ovarian tissue. Both medulla and cortex are perfectly distinct, as well as sections of the oviduct carried over into the male at the time of operation. There are many Graafian follicles of various stages of growth and one is able to follow the follicular history from the primordial follicle through its various stages, including follicles whose cavities are just appearing, follicles with well-formed cavities and perfectly distinct discus proligerus with its enclosed ovum, follicles undergoing atresia in which the cavity is large, or those having progressed so far that only the indication of the old follicular cavity remains. These masses are surrounded by the connective-tissue stroma and the granulosa cells have reverted to a lipoid-containing stage characteristic of the interstitial cells; a very great number of this character are present.

This graft is the smaller of the two and consists of but about ninety sections.

Graft no. 2. The ovarian tissue proper of this graft (after a residence in the male of 232 days) is composed of about 230 sections (cut  $10\mu$  thick). The tissue, which is accompanied by a small piece of the oviduct, is characteristic in most of its structures. Medulla and cortex are distinct, the stroma is characteristic, and all stages in the life-history of graafian follicles are abundantly represented. By actual count, forty-six normal



follicles containing normal-appearing ova with distinct and well-stained nuclei have been studied, besides numerous atretic follicles containing ova in which there is a spindle bearing perfectly distinct chromosomes, ova that are not surrounded by a discus proligerus, but are free within the follicular cavity and undergoing fragmentation, as well as all stages of degeneration of the follicle and its conversion into interstitial tissue. Figures 3, 4, 5, 7, 8, and 11, all from this graft, represent the development of a follicle and the fate of the follicles undergoing atresia. Figures 7 and 8 show atretic follicles containing an ovum with a mitotic spindle or fragments of the ovum.

There is an abundance of very small follicles (primordial follicles) with or without ova, but there is no evidence of a corpus luteum or of ovulation.

Testis. The remaining testicle of this male, which had been functioning as a male, is perfectly normal. A section of it is shown in figure 15 and it warrants no further consideration.

*Animal 49 C.* Male rat, born Nov. 6, '17. Dec. 3, the right testis was removed, and two pieces of ovary from sister placed subcutaneously. Aug. 22, '18, animal killed, the left testis was normal, and both ovary grafts had persisted for the period of 230 days; both grafts sectioned.

Graft 1. This graft, the smaller of the two, consists of about 165 sections; cortex and medulla are distinct and between sixteen and twenty follicles containing well-defined ova surrounded by the discus proligerus and containing a well-stained nucleus have been studied. These follicles represent all stages of development from the primordial follicle to the follicle ready for ovulation. Scores of atretic follicles of all sizes are present; these comprise follicles with fragmenting ova, follicles whose contained ovum appears normal, but in which the discus proligerus has undergone partial or almost complete dissolution, and smaller-sized follicles that are distinctly surrounded by a fibrous-like connective tissue, but whose granulosa cells are enlarged and show the loose reticular cytoplasm characteristic of the cells containing considerable lipoid materials and in which there is either a well-defined follicular cavity, a very small cavity, or again masses of the same character in which no cavity is dis-

cernible; these latter masses are the characteristic groups of interstitial cells. A considerable amount of the fimbria of the oviduct accompanies the section of the ovarian tissue.

Graft 2. This section, somewhat larger than the previous one, consists of about 300 sections of ovarian tissue. Here also the medulla and cortex are characteristic, and some twelve to fifteen normal follicles are present as well as scores of all sizes of atretic follicles. Figure 6 shows one follicle whose ovum has just completed the formation of a polar body; the ovum is yet surrounded by the discus proligerus though this is apparently undergoing dissolution. Sections of the oviduct accompany this graft.

There is no indication either of corpora lutea or of ovulation in these two grafts.

*Animal 51 D.* Male, born Feb. 27, '18. Operation Apr. 13, one testis removed (one undisturbed); two small pieces of ovary from sister placed subcutaneously and intraperitoneally. Aug. 22, '18, killed; the remaining testicle was normal as well as sperm sacs; both ovarian grafts had persisted for 130 days; both grafts sectioned.

*Microscopic examination.* Each of the grafts has suffered a considerable amount of resorption and around the edges of the ovarian tissue there is distinct evidence of degeneration and connective-tissue proliferation.

Graft no. 1 consists of but sixty or sixty-five sections of ovarian tissue, but within this small amount there are several distinct follicles, a few of the smaller ones apparently normal, but the larger ones atretic.

The especial interest attached to this graft is the presence of two distinct and separate bodies of cells; these cells are larger than the cells found in any other part of any of the graft material, and they appear to represent unmistakable, though small, corpora lutea. They are somewhat removed from the surface of the graft and are surrounded by stroma cells.

If these are small corpora lutea (and there seems nothing to indicate the contrary) the question of their origin presents itself. As has been repeatedly mentioned, no structure or condition in any of the grafts has given the least evidence that ovulation has

occurred and it seems possible, in the absence of other indications, to suppose this a case of corpus luteum formation without ovulation; and if this assumption is correct, evidently the cells of the granulosa layer have been converted into the cells of the corpus luteum. This idea is not without a parallel, for evidences of somewhat similar conditions have been reported (see work of Rasmussen and Corner).

Graft no. 2 presents nothing worthy of comment except its small size (about forty-five sections of ovarian tissue) and the presence of a comparatively large number of follicles most of which are small in size, and the majority of them atretic.

*Animal 51 E.* Male rat, born Feb. 27, '18. Apr. 13, one testis removed and two pieces of ovary from sister placed subcutaneously and intraperitoneally. Aug. 22, '18, killed; remaining testis normal; both grafts persisted for the 130 days; both grafts sectioned.

Graft 1. The tissue of this graft proves to be only a mass of the oviduct; several sections in one plane show that it has been greatly coiled. Practically the entire mass of ovarian tissue has been resorbed.

Graft 2. This graft has been well preserved and consists of about 290 sections of ovarian tissue. Medulla and cortex are distinct and fifty-five normal follicles have been studied (follicles containing a normal-appearing ovum with a distinct and well-stained nucleus); besides the normal follicles there are literally hundreds of follicles that are in a stage of atresia. These atretic follicles may contain ova showing a distinct spindle with chromosomes arranged equatorially, ova that have undergone fragmentation, or the follicle may contain no ovum at all, but yet show a distinct and large follicular cavity lined by an uninterrupted granulosa layer of several cells deep; or the cavity may be small with a distinct granulosa layer, the cells of which have been changed to the large lipid cells; or yet the cavity may have been entirely obliterated, but the large lipid cells are a distinct mass and are surrounded by the fibrous layer that represents the old theca externa, the mass now being regarded as interstitial cells.

Figure 2 is a section of this graft and the relative number of atretic follicles to normal ones is clearly shown; a and b are



normal follicles as b shows a normal-appearing ovum farther along in the series in which the nucleus is distinct and well stained; the ovum is contained within the discus proligerus. Three ova in this graft contain a mitotic figure (polar spindle).

*Animal 51 G.* Male animal, born Feb. 27, '18. Apr. 13, '18, one testis removed and two pieces of ovary from sister grafted subcutaneously. Aug. 22, killed; the remaining testis was normal, and both ovarian grafts had persisted for the 130 days; both were sectioned.

*Microscopic examination.* These two grafts present no features of note that have not been illustrated by the previous grafts, except the presence of ovarian tissue in the midst of a considerable amount of degeneration in the implanted grafts. Graft 1 consists of about 275 sections of ovarian tissue, but this is represented almost entirely by a strip of cortical tissue restricted to one side of the recovered graft. The remainder of the graft is principally oviduct. Within the ovarian tissue, however, there are some nine or ten Graafian follicles that contain ova with well-stained nuclei. Aside from these few normal follicles, there are numerous atretic follicles and considerable masses of interstitial cells.

In graft no. 2 there is more degeneration than in the previous graft, yet about the same number of normal follicles. Here also the number of atretic follicles is very great. In neither of these grafts is there evidence of ovulation or of corpus luteum formation.

*Animal 51 H.* Male, born Feb. 27, '18. Apr. 13, one testis removed and two pieces of ovary from rat of same age placed subcutaneously. Aug. 22, '18, killed; the remaining testicle was normal and both ovarian grafts had persisted for the 130 days; only one was sectioned.

*Microscopic examination.* The principal part of this graft is oviduct. The ovarian tissue is restricted to a very small area to the side of the oviduct. The tissue consists of but thirty-five to forty sections and is of a width that would accommodate about two half-sized follicles. However, within this very small mass of tissue there are three follicles that are practically normal; they contain ova whose nucleus is normal and distinct, but the

granulosa is beginning to show an abnormal condition. In this, as well as in all other graft sections of the entire series, the interstitial cell masses are relatively abundant.

### *B. The testis*

*Animal 40-4 A2B2 III.* Female, born Nov. 14, 1917. Dec. 18, right ovary removed, two small pieces of testis from a brother rat were grafted subcutaneously. May 18, '18, animal killed; left ovary normal, oviduct, and uterus normal, both grafts persisted, one sectioned.

Serial sections of the graft show the testis material imbedded between layers of muscle tissue. During the healing of the wound after the operation, connective tissue grew into and around the graft so that at first sight it presents the appearance of a loosely arranged connective tissue within which are located the conspicuous seminiferous tubules. The tubules are quite numerous, the number of sections varying as the majority of the tubules are cut in cross or longitudinal sections.

Figure 13 is a reproduction of a portion of one section and shows the characteristics of the degenerate tubules; this tubular mass is continuous in 420 sections of the graft taken from the female animal. Of the tubules themselves, besides the connective-tissue framework, there are a few relatively large cells inside the tubular walls scattered indefinitely amid a reticular, non-cellular material; these are the cells that are commonly spoken of as the remaining Sertoli cells.

The intertubular spaces are occupied by connective tissue, the two kinds of foreign cells mentioned previously, and some interstitial cells. The chief feature to consider, however, is that there is no evidence of a hypertrophy of the interstitial gland of the testis in any of these grafts, but one must consider that the tubular mass is not enclosed by its tunica albuginea (only a small piece of the testicle having been used in the transplantation), hence the interstitial cells, not being confined, would probably have been more easily scattered by the ingrowth of connective tissue. It may be that this difference may account for the difference in results of these and Steinach's graft material, in reference to the quantity of interstitial cells, and that in

Steinach's material there was an apparent hypertrophy rather than a real one; had the entire mass of interstitial cells of the original testis been concentrated into such a small mass as would have remained after degeneration of the germinal epithelium, they would have appeared in considerably more concentrated masses than in the normal condition.

*Animal 40-4 A2B2 V.* Female, born Nov. 14, 1917. Dec. 17, right ovary removed, two testis grafts placed subcutaneously. Sept. 2, '18, killed; left ovary normal, both testis grafts had persisted (this female gave birth to a litter of five on May 10). Grafts were not sectioned.

*Animal 49 B.* Female, born Nov. 6, 1917. Dec. 1, right ovary removed and two pieces of testis from brother rat placed subcutaneously. Aug. 22, '18, animal killed; left ovary normal, both testis grafts had persisted, good vascularity in both. Both grafts sectioned, as well as ovary.

Graft 1 consists of a very considerable mass of typically degenerated seminiferous tubules and interstitial material. Figure 14 is a high-power drawing of a small part of one section, and shows five or six tubules in cross-section with the accompanying interstitial material between the tubules. It can readily be noted that the tubules are not normal, but that during their residence in the female host the germinal epithelium has suffered destruction; all that remains within the tubules of a cellular nature are the large cells lying close to the basement membrane and considered as the Sertoli cells.

Graft 2, in comparison with the preceding one, is smaller in size, but the tissue presents no new features worthy of note. Seminiferous tubules are present in 200 or more sections of the graft tissue and these are well rounded and show but little tendency to collapse. The entire mass of the graft is surrounded by muscle tissue.

Sections of the remaining ovary of this animal show it to be entirely normal, though the animal had never given birth to a litter.

*Animal 51 A.* Female, born Feb. 27, '18. April 16, one ovary removed, testis from brother implanted subcutaneously and intraperitoneally. Aug. 22, '18, killed; right ovary normal, small intra-



peritoneal graft, but no subcutaneous graft (female had given birth to a litter). Tissue not sectioned.

*Animal 51 B.* Female, born Feb. 27, '18. April 16, one ovary removed, testis from brother grafted subcutaneously and intraperitoneally. Aug. 22, '18, killed (suckling litter when killed); right ovary normal, both grafts persisted.

Graft 1 consists mostly of scar tissue and connective tissue. The greater part of this graft has been resorbed, but here and there are the distinctly well-rounded, degenerating seminiferous tubules containing only the Sertoli cells and the reticular network. The tubules are not collapsed and but little if any interstitial tissue is present.

Graft 2 likewise contains but little of the original testis tissue implanted. The tubules present are similar to the tubules in all the grafts so described, whether the remaining tissue is abundant or consists only of a few scattered tubules. Nothing beyond what has been previously described is worthy of note.

*Animal 51 C.* Female, born Feb. 27, '18. April 13, left ovary removed, testis from brother placed subcutaneously and intraperitoneally. Aug. 22, '18, killed; right ovary normal, both grafts persisted (had given birth to a litter).

Graft 1 consists of an elongated strand of seminiferous tubules that are present in 250 sections; some cross-sections will show only four or five sections of these tubules, while others may show as many as fifteen or twenty sections of the tubules. These merit no especial description, as they differ from the others in no essential features.

Graft 2 for the greater part has been resorbed. Here also the remaining tubules are imbedded in the muscular or connective tissue and are entirely similar to the tubules of all other grafts.

## VI. DISCUSSION

After a consideration of such a group of grafts as those described, it is perfectly evident that pieces of ovaries and testes can be successfully grafted into an animal of the opposite sex possessing one of its normal sex glands, and that these grafts may persist for a period of eight and one-half months in a normal

condition (at least in the case of the ovary), *a very long time in proportion to the length of life of the animal*. And not only does the ovary obtain enough vascularization to persist, but it also proceeds in its most obvious function—that of the development of the ovum from the primordial-follicle stage to the stage of maturation or formation of polar bodies; this process of development is going on in a male animal that is functioning as a male with one normal testis present. The demonstration of such a set of facts proves the possibility of a functional hermaphroditic condition in mammals.

A question may arise as to the actual functional condition of the ovarian graft, but beyond a doubt the most apparent function of the ovary—that of affording a suitable environment in which the germ cells can grow and become mature—has been adequately demonstrated; failure of ovulation is of secondary importance.

The writer is entirely unable to account for the consistently negative results obtained by Steinach. On reading his papers one concludes that this investigator has conducted many operations of a similar nature to those described in this paper, though it is very difficult in many cases to ascertain the extent of his actual manipulations. He consistently maintains that a secretion from the interstitial cells of a sex gland (Pubertätsdrüse) acts in a double capacity—that of promoting the somatic and psychical characteristics of the animal whose sex is represented by the graft, and at the same time inhibiting the characteristics of the opposite sex. My own observations do not substantiate his assertions that an ovarian graft in a male animal will inhibit the growth of the testis or other masculine structures and the psychical nature of the animal; or that a testicle or testis graft prevents the growth of an ovary or inhibits the function of the gland. In a male animal with one testicle intact and uninjured, ovarian grafting has been so successful that both ovarian grafts have persisted for a period of eight months (animal 49 A) in a condition that must be regarded as functional; and these two comparatively large ovarian grafts, though present for this relatively long period, have had no apparent inhibiting effect

upon the penis, testis, sperm sacs, or psychical nature of the animal; for it has been functioning as a male animal, having been used for breeding, and histological sections show that the seminiferous tubules are actively producing spermatozoa.

In the light of many researches, it is wrong to assume that these grafts, on account of their being smaller than the normal gland, are not able to exercise their full function. The work of Pezard, Stotsenburg, Sand, Steinach, and others leads one to assume that even a small functional part of one sex gland is able to maintain the typical somatic and psychical characters of the animal represented by the small graft.

Many of the grafts described in section V have evidently suffered considerable degenerative changes and many of the transplants have been entirely resorbed. This, however, is true in many autoplasmic grafts, and hence cannot be accounted for by assuming that one sex gland secretes a substance that inhibits the growth of the opposite one. If a testicle and ovarian grafts exist in the same animal in a functional condition for a period of approximately one-fourth or one-fifth the entire natural sexual life of the animal, one cannot assume that a secretion from each gland causes a destruction of the other.

Sand states that he was entirely unable to obtain growth of a subcutaneous transplant in an animal of the opposite sex if the sex glands of the latter were present. He develops quite an elaborate hypothesis to explain the negative results, but again, my own observations indicate that no such hypothesis is necessary, for positive results have been obtained in many cases.

In the entire series of grafts, with one possible exception, there are no indications that ovulation has occurred, and it is interesting to consider the possible factors involved. Leo Loeb determined that by underfeeding female guinea-pigs sterility could be produced. Under the conditions of the experiment, the follicles behave in a manner somewhat similar to the follicles in the grafts of the rat ovary; instead of proceeding in their normal development, they become atretic. It may be possible that in the new environment in which the transplanted ovary is placed, vascular connections are not entirely sufficient to make



it possible for the completion of the process of ovulation, though this seems scarcely probable. Abnormal pressure may have an effect in abolishing ovulation, for obviously in its strange environment the graft, very often surrounded by muscle tissue, would be subjected to a greater pressure than it would in its normal peritoneal surroundings. It seems more probable, however, that the proper physiological correlation of unknown factors leading up to and initiating ovulation in the normal female is not present in the male animal in which the graft is located. We meet, likewise, a similar condition in young females preceding the onset of sexual maturity.

In a female rat thirty-six days after birth the ovary contains many large follicles that, in the adult female, we would class as mature follicles, but the rat does not become sexually mature until seventy to ninety days after birth. Previous to this time, the follicles do not undergo ovulation, judging from the tremendous number of atretic follicles (fig. 12), but the follicles degenerate as do those described in the grafts. In one case as in the other we can assume an absence of physiological correlation necessary for the phenomenon of ovulation, but whether the seat of such influences may be within the ovary itself or in some other endocrine organ is problematical. Long and Quisno ('16) maintain that female rats isolated from males will ovulate about every ten days, but in the grafts ovulation has evidently been almost, if not entirely, suppressed; the ovum, instead, is thrown out into the follicular cavity upon dissolution of the discus proligerus, and there undergoes fragmentation and disappears while the stratum granulosum is converted into a mass of large cells containing lipoid material, that are present between the developing follicles.

One point however is perfectly apparent, *the presence of a normal testicle does not prevent the persistence or growth or development of an ovary grafted into a male rat if this graft is made early in the life of the animal.* This fact established, how are we to harmonize it with the facts made known by Lillie in the case of the free-martin? If the blood of a male and female pair of cattle twins intermix during intra-uterine development, the

ovary does not develop normally. Theoretically, a hormone from the male is the cause of the abnormal condition, while in the present case the two sex glands coexist without any apparent ill effect to either. In the case of the free-martin, however, there is no real indication of an antagonism between the sex glands. Hormone action is not characterized by an inhibition, but by a stimulation, and the stimulus from the male hormone acting upon the indifferent gonad of the foetus exerts an influence quantitatively greater than the inherent influence toward femaleness, hence the resultant of the two forces is a type of development more nearly resembling the male than the female.

Somatic development is undoubtedly influenced very greatly by the presence or absence of a sex gland, as shown by numerous experiments, but the sex gland is not alone responsible for all the differences that occur. There is also that unknown influence which in the first place determines whether the indifferent stage shall, under normal conditions, progress toward maleness or femaleness. But however potent a hormone of an embryo or adult may be in modifying the somatic or psychological nature of the animal, the two hormones acting in the same adult individual give no evidence of antagonism as Steinach maintains. The conditions in pseudohermaphroditic mammals may then be conceived of as the resultant of two opposing stimuli—opposing not in the sense of inhibition, but merely that the two influences tend to lead development in two directions. The degree of intermixture of the sexual apparatus would thus be a function of the quantitative difference between the two stimuli and not the result of the suppression of one gonad by the other.

## VII. SUMMARY AND CONCLUSIONS

1. In the rat, an ovary grafted into a male animal, possessing one normal testicle, will become vascularized and grow for at least eight and one-half months after the transplantation.

2. The graft, after remaining in the male for this relatively great length of time, possesses all the characteristic structures of the ovary excepting the corpora lutea.

3. The characteristic growth of graafian follicles continues in a perfectly normal way up to about the stage of maturation; from this time on the history of the follicle is abnormal.

4. From about the stage of maturation the follicles undergo atresia, the ovum fragments and disappears, and the cells of the stratum granulosa are converted into interstitial cell masses.

5. The large ovarian grafts, containing all the characteristic structures of the ovary excepting the corpus luteum, have given no evidence of a deleterious influence upon the male somatic or psychical characteristics.

6. Pieces of testis grafted into a female animal, possessing one ovary, persist at least eight months.

7. The seminiferous tubules, as in most cases of testis grafts, are present, well rounded, but contain only Sertoli cells; the germinal epithelium has undergone degeneration.

8. The presence of the testicular grafts has no deleterious influence upon the somatic or psychical characteristics of the female animal.

9. There is no indication of an antagonism between the ovary or testis, even when existing in the same animal in a functional condition.

10. These experiments afford direct evidence of the physiological possibility of a functional hermaphroditic condition in mammals.



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Un estudio experimental de los quimorreceptores tarsales de dos  
mariposas ninfálidas.

Las mariposas ninfálidas *Pyrameis atalanta* Linn. y *Vanessa antiopa* Linn., responden a la estimulación química desarrollando la trompa. Mediante esta respuesta ha sido posible localizar y estudiar algunos órganos del sentido químico, desconocidos hasta el presente, en estas mariposas. Estos órganos están localizados en los tarsos de las cuatro patas ambulatorias y, aunque su distribución exacta no ha sido determinada, los experimentos llevados a cabo por el autor demuestran que existen por lo menos en la parte del tarso que comprende el extremo distal del segmento proximal y los cuatro segmentos distales.

El las dos especies estudiadas los quimorreceptores tarsales permiten a los animales distinguir el jugo de manzana del agua destilada, aun cuando son sensitivos a ambas sustancias. Experimentos adicionales en *Pyrameis* han demostrado que esta especie puede también distinguir una 1 M. solución de sacarosa del agua destilada, y soluciones tales como 1 M. HCl, M/600 de sulfato de quinina, y 1 M NaCl, del agua destilada o de la sacarosa. Una función muy importante de los quimorreceptores tarsales, por consiguiente, está relacionada con las sustancias alimenticias y el agua. No solo el animal puede distinguir la presencia y posición exacta de dichas sustancias en el substrato, sino que también puede distinguir su naturaleza en cierto grado.

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# AN EXPERIMENTAL STUDY OF THE TARSAL CHEMO- RECEPTORS OF TWO NYMPHALID BUTTERFLIES<sup>1</sup>

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SIX FIGURES

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## INTRODUCTION

Any adequate investigation of the normal physiology of chemo-reception among insects is dependent upon two conditions. First, the animal when stimulated must evince some clear and unmistakable response of constant nature. Second, this response must be evoked by stimuli encountered by the animal in its normal environment. It is not necessary to confine experimentation entirely to such stimuli. Conclusions as to the normal functioning of the sense organ, however, can only be made with certainty when it has been demonstrated that such stimuli do produce a clearly discernible response.

<sup>1</sup> I wish to take this opportunity to thank the authorities of Miami University for the use of their laboratories during the summer of 1919. Through this generosity, the work on *Pyrameis* was accomplished. I wish also to express my thanks to Harvard University for the grant of a Parker Traveling Fellowship for 1917-1918, which made possible the work done on *Vanessa*. This work was carried out in the Zoölogical Laboratory of the University of California, and to the departmental staff there I am indebted for many courtesies.

The nymphalid butterflies, *Pyrameis atalanta* Linn. and *Vanessa antiopa* Linn., meet these experimental requirements in an admirable way. They may often be observed, in company with one or two other species of nymphalids, hovering about a tree trunk, where they frequently alight and remain, wings closed, for considerable intervals of time. Investigation will show that the portion of the trunk visited has been injured in some way and that the butterflies alight there to feed upon the exuding sap. Similar feeding activities may be observed about orchards in the autumn, when fallen and decaying fruit, such as apples and pears, attract the animals. *Pyrameis* and *Vanessa* thus respond to food substances by uncoiling the proboscis. This response—the proboscis response, as I shall term it—is an absolutely clear one, for in the unstimulated animal the organ remains compactly coiled against the head. Consequently, a partial extension of even slight magnitude is readily observed, while a complete extension with the subsequent probing of the substrate is unmistakable. Moreover, fruit juices, which evoke this response in the natural environment, are easily obtainable.

Chemoreceptors may be divided into two classes: first, those affected in general by volatile materials, the source of which may be more or less remote from the receptive surface; second, those affected in general by non-volatile materials, the source of which must be in intimate contact with the receptive surface. The former serve as distance chemoreceptors; the latter, as contact chemoreceptors. In the last analysis both are stimulated by a solution of the exciting material, the solvent consisting, at least in part, of the secretion present on the sensitive surface. The above distinction, therefore, far from being an absolute one, is merely useful as the best single condition by which the two groups of sense organs may be conveniently differentiated. Following the objective nomenclature, the appropriate stimuli for these two classes of sense organs may be designated, respectively, as distance chemical stimuli and contact chemical stimuli. For brevity, however, it will frequently be useful to omit the qualifying term, chemo or chemical, understanding that in the present paper the discussion is limited to organs of chemical sense.

By means of the proboscis response, it has been possible to study both classes of chemoreceptors in *Pyrameis* and *Vanessa* in a most satisfactory way. In the present paper, however, we shall confine our attention to certain of the organs of contact reception. These organs are apparently of considerable importance, and yet, as far as I am able to ascertain, they have completely escaped observation heretofore. Their morphology and their general occurrence among insects are now being investigated and will be reported upon subsequently.

#### LITERATURE

The organs of chemical sense in insects have been the subject of a voluminous literature. Most of this work, however, has been confined to the organs of olfaction, distance chemoreceptors, while the organs of taste, contact chemoreceptors, have received relatively little attention.

Investigators have differed widely as to the exact location of the olfactory organs. In connection with the data to be presented later, it is interesting to note that certain organs located in part on the leg have been thought by some to function as olfactory organs. Attention was first directed to these organs by Hicks ('60). Recently McIndoo ('14 a, '14 b, '14 c, 15, 17) has made a very comprehensive study of the same organs in a number of different groups of insects and is strongly convinced that they are olfactory in function. Concerning the exact location of these organs in *Lepidoptera*, he says ('17, pp. 40-41):

The disposition of the pores on the trochanters and femurs of a few of the species is similar to that of the honey bee, but only occasionally are pores found on the proximal ends of the tibiae and never on the tarsi, as observed in the *Hymenoptera*. A few pores, usually near the distal ends of the tibiae, were seen in 21 of these specimens . . . . , and pores were observed in the tibial spines of 12 individuals. . . . .

Among the butterflies studied was *Vanessa antiopa*, but in this form McIndoo found no pores distal to the proximal end of the femur, where isolated pores occurred. As will be shown later, this is a very different location from that occupied by the organs we are to discuss. There is thus no possibility that the organs



described by McIndoo are the ones concerned in my own experiments.

As stated above, the sense of taste in insects has received relatively little attention. Comprehensive reviews of the existent literature may be found in Packard ('98), Forel ('07), and Berlese ('09). It is sufficient for our purpose to call attention to two facts. First, experiments such as those of Will ('85) and Forel ('07) have shown rather conclusively that in at least some insects there is a well-defined sense corresponding to the sense of taste in man. Second, the organs which have been described as mediating this sense are organs which have been found on or near the mouth parts or within the buccal cavity. As Will ('85, p. 685) and Forel ('07, p. 104) have pointed out, however, it is virtually impossible to demonstrate experimentally that these organs are certainly gustatory in function, since their removal results in an inability to take food. It has been necessary, therefore, to deduce their function from the evidence afforded by their location and structure.

#### MATERIAL AND METHODS

The experiments on *Pyrameis* were all carried out at Oxford, Ohio. The specimens employed in the first few experiments were animals which were taken in the field early in the spring. A few of these animals had undoubtedly passed the winter as adults, but the very fresh appearance of the great majority of specimens showed clearly that they had just emerged from the pupae. In all subsequent work, however, specimens were used which had been reared in captivity. The age and history of each butterfly were thus known, and it was possible to select animals of approximately uniform age and state of inanition for a given experiment. The work on *Vanessa* was done at Berkeley, California. All of these animals were captured specimens. They were obtainable in numbers on and about the campus of the University of California from the middle of October until the middle of November. A few individuals were taken by hand while feeding on flowers, but as a rule a net was necessary, for this species is easily disturbed and flies rapidly.

In the laboratory the butterflies were housed in large cages, the largest being 1 m. x 1 m. x 1 m. These were constructed of light wooden framework, covered with mosquito bar. Under these conditions, *Pyrameis* lived from three to four days without food or water. This period undoubtedly depends much upon temperature and the consequent rate at which water is lost through evaporation. During these experiments the weather was extremely warm and the windows of the laboratory were kept open. At a lower temperature the less rapid loss of water would probably increase the longevity considerably. Properly fed, *Pyrameis* survived longer, but needed to be fed regularly every few days in order to survive in good condition. *Vanessa*, however, proved a much hardier animal, surviving a period of ten days or even considerably longer without food or water.

Individual butterflies were easily distinguished by clipping the wings slightly, in different ways, and numbering the animals accordingly. A spring clothes-pin, from which the beveled tips had been cut off, was employed as a holder to handle the animals. This served the purpose well, holding the animal firmly and at the same time permitting easy manipulation.

The grip of the holder on the wings, however, together with the manipulation involved in placing the animal in the holder, induced the death feint in both species. In the case of *Pyrameis* this reaction was not very pronounced, and even in the most extreme cases it was sufficient to bring the feet in contact with a substrate a few seconds for the animal to recover completely. Once the death feint had worn off, moreover, the butterfly remained active and responsive.

With *Vanessa*, however, the death feint was prolonged and sometimes very difficult to overcome. It was found that recovery could be hastened by gently dragging the feet of the animal over a surface, and this was done prior to each trial. But even after this procedure, the removal of the animal a short distance to the place where it was to be tested often induced more or less of the death feint again. Whenever an animal failed to respond to chemical stimulation in the first part of a trial, therefore, it was gently lifted and set down several times in succession,

in the hope of counteracting the possible effect of the death feint. I am certain, however, that with all these precautions, the death feint together with the effect produced by holding the animal accounted for many failures to respond to chemical stimulation. This opinion was strengthened by the fact that several animals which had failed to respond during a trial did so immediately upon being released from the holder.

Since the data to be presented in this paper have been obtained solely through a study of the proboscis response and the conditions which effect it, a more detailed account of this reaction is necessary at this point. In a few instances the proboscis was completely uncoiled only to be recoiled shortly afterwards. As a rule, however, once the organ was extended, the butterfly actively explored the substrate with it, and upon contact with the stimulating substance began to feed. The animal was generally removed as soon as the proboscis was completely extended, in order that feeding might not interfere with the sensitivity to food substances. So strong was the response, however, that frequently, as the animal was being carried back to the cage, the proboscis would remain extended and continue to probe the empty air in a vigorous manner.

In many cases the extension of the proboscis was not complete. The degree of these partial extensions varied from slight, and occasionally, barely visible jerks to almost complete extensions. These were easily observed in the great majority of cases, and indeed it was only in a few trials of a very few individuals that the barely visible jerks were noted. Frequently the proboscis was partially uncoiled and, like the hair-spring of a watch, was kept springing back and forth for some seconds. Occasionally, this culminated in a complete extension, but more often it was followed by a complete recoiling of the organ.

The duration of each trial was one minute, unless the proboscis was completely extended before that interval had elapsed. In the latter case, as stated above, the animal was immediately removed to prevent feeding. If there occurred any visible movement of the proboscis whatever during the trial, it was counted as a response. If, on the contrary, there was no visible



movement of the proboscis during one minute, a 'no response' was recorded. With the 'no response' class, I have also included a few questionable cases, in which I could not be certain whether very faint movements had occurred or not. All such cases have been indicated in the tables by interrogation marks.

#### THE PRESENCE OF CHEMORECEPTORS IN THE TARSI

##### *1. Apparatus*

The results of preliminary observations and experiments soon convinced me that there were present in the anterior walking legs of *Vanessa* and *Pyrameis*, sense organs, the stimulation of which evoked the proboscis response. To test this more fully, the apparatus shown in figure 1 was constructed. It consisted of a small wooden platform, *ww'*, bearing two wooden cross-pieces, *xx'* and *yy'*, which supported a tightly stretched wire screen, *s*. The dimensions of this construction were such that a glass Petri dish, *p*, 15 cm. in diameter and 1 cm. deep, could just be slipped under the screen without contact with it. In the Petri dish were mounted two small rectangular pans of tin, *a* and *b*. These were 4 cm. long, 2 cm. wide, and of such height that their top edges were on a level with the rim of the Petri dish. Two rectangular openings were cut in the screen, so that when the Petri dish was in place they were directly over the pans. Except these two places, the Petri dish was everywhere covered by wire screen.

Preliminary to each experiment, the apparatus was placed on a tripod, on a table, and the Petri dish was filled with applejuice.<sup>2</sup> Two packs of cheese-cloth were folded to fit the pans. One of these was saturated with applejuice and placed in pan *a*; the other, saturated with distilled water, and placed in pan *b*. In each case the exposed surface of the pack was carefully

<sup>2</sup> Several of the bottled preparations of applejuice were used in the course of these experiments. In the first part of the work on *Vanessa* I continued to use from an opened bottle for several days, so that fermentation had set in toward the end. In all subsequent experiments, however, a fresh bottle was opened each day.

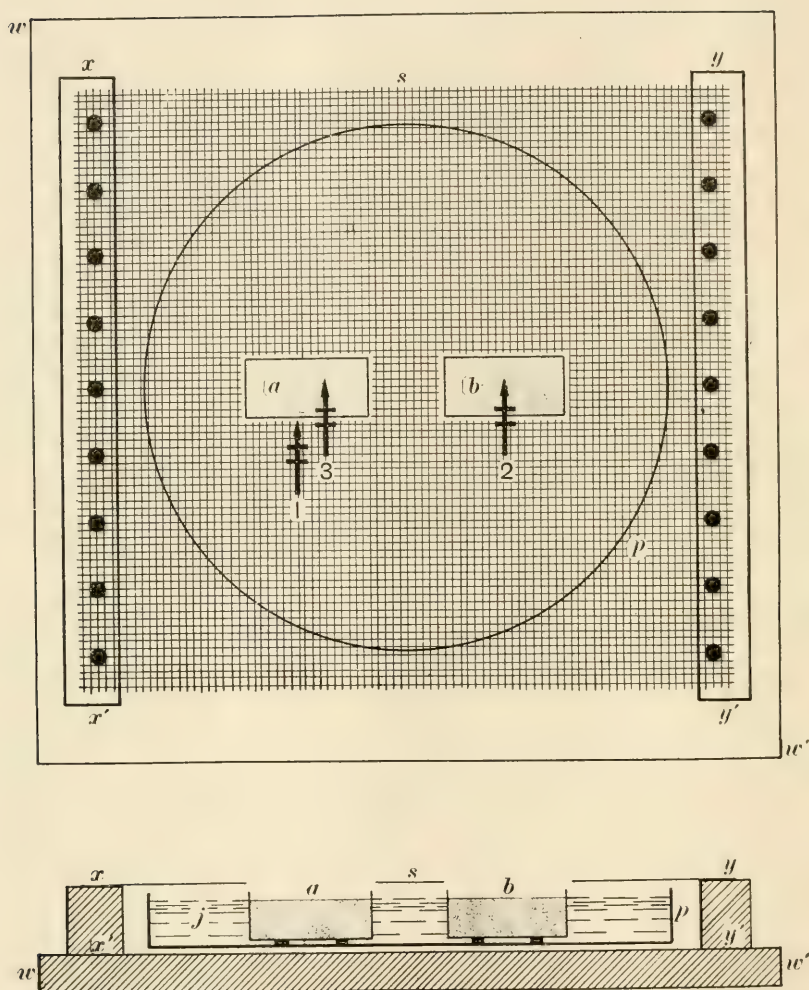


Fig. 1 Plan of apparatus as seen from above and as seen in cross-section. *a*, rectangular pan of tin, containing cheese-cloth pack saturated with applejuice; *b*, rectangular pan of tin containing cheese-cloth pack saturated with distilled water; *j*, applejuice; *p*, Petri dish; *s*, wire screen; *ww'*, wooden platform; *xx'* and *yy'*, wooden cross-pieces. The arrows, 1, 2, and 3, represent the positions in which the butterflies were tested, the position of the walking legs being shown by the cross-bars.

adjusted so that it was on a level with the surrounding applejuice in the Petri dish.

A butterfly was removed from the cage and placed in the holder. When the death feint had worn off, it was tested in one, two, or three different positions on the apparatus (fig. 1, 1, 2, 3).

In the first position, the animal was held as near the edge of pan *a* as possible, the antennae reaching out over the cloth saturated with applejuice, but the four walking legs on the screen. (It will be recalled that among the *Nymphalidae* the anterior pair of legs is rudimentary and subserves no locomotor function.) In this position, therefore, the chemical stimuli received by the animal consisted of distance stimuli only, viz., the vapors given off by applejuice.

In position 2 the animal was also subject to distance stimuli. Qualitatively these were the same as in position 1, and quantitatively they could not have differed greatly, since pan *b*, like pan *a*, was everywhere surrounded by a relatively large area of applejuice. But to these distance stimuli was added whatever stimulation might be afforded by the contact of a portion of the second tarsi (anterior ambulatory) with the cheese-cloth pack saturated with distilled water. Naturally, the number of tarsal segments in contact with the water-soaked substrate varied somewhat from time to time, depending on the posture of the entire leg.

Position 3 bore the same relationship to pan *a* that position 2 did to pan *b*. The animal was thus subjected to the same distance stimuli as in the two previous positions, but the second tarsi were brought into contact with a cloth substrate saturated with applejuice.

The conditions of chemical stimulation in the three positions above described may be summarized as follows:

Position 1. Distance stimuli from applejuice.

Position 2. Distance stimuli from applejuice plus contact stimulus of distilled water on second tarsi.

Position 3. Distance stimuli from applejuice plus contact stimulus of applejuice on second tarsi.



## 2. Experiments on *Pyrameis*

In experimenting with *Pyrameis*, specimens were first tested in position 1, then in position 2, and finally in position 3 (fig. 1, 1, 2, 3). The trials in the three positions were not made in immediate succession, however, but separated by a minimal interval of fifteen minutes. Two experiments, composed of three sets of trials each, were carried out. The total number of butterflies employed was eight. In both experiments the specimens were collected in the field. In experiment 1 the

TABLE 1  
*Pyrameis*

NUMBER OF EXPERIMENT	DURATION IN DAYS	NUMBER OF ANIMALS	POSITION 1			POSITION 2			POSITION 3		
			No response	Response		No response	Response		No response	Response	
			No extension of proboscis during 1 minute	Partial extensions of proboscis during 1 minute	Complete extension of proboscis in 1 minute or less	No extension of proboscis during 1 minute.	Partial extensions of proboscis during 1 minute	Complete extension of proboscis in 1 minute or less	No extension of proboscis during 1 minute	Partial extensions of proboscis during 1 minute	Complete extension of proboscis in 1 minute or less
1	$\frac{1}{2}$	5	10 + 2?	2	1	11 + 1?	2	1	0	0	15
2	$\frac{1}{2}$	3	4 + 1?	3	1	8	1	0	0	0	9
Totals		8	17	7		20	4		0	24	
Per cent . .			71	29		83	17		0	100	

animals were collected in the evening and tested the following morning; in experiment 2 they were collected and tested in the same morning. The results of these experiments are presented in table 1 and figure 2.

It will be noted (table 1) that while only 29 per cent of the butterflies responded in position 1 and but 17 per cent in position 2, 100 per cent responded in position 3. Several facts are brought out clearly by these data. *Pyrameis* does respond to the distance stimuli afforded by applejuice in a number of cases. The number of these responses is not increased by bringing the second tarsi in contact with distilled water. But if the second tarsi

are permitted to come in contact with applejuice, the number of responses is enormously increased. In other words, in a large number of cases in which the butterflies do not respond either to distance stimuli or to distance stimuli plus the contact stimulus

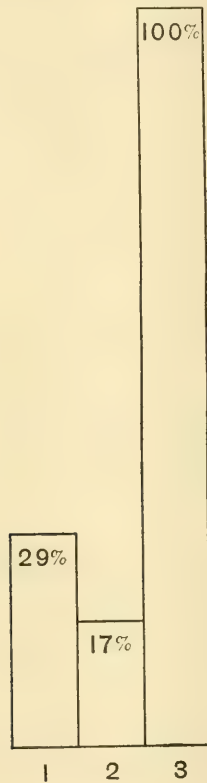


Fig. 2 Graph showing the percentage of responses obtained from specimens of *Pyrameis*, when subjected to the same number of trials in positions 1, 2, and 3 on the apparatus shown in figure 1.

of distilled water on the second tarsi, they do respond to distance stimuli plus the contact stimulus of applejuice on the second tarsi. Clearly, therefore, the second tarsi are sensitive to contact chemical stimulation and must contain appropriate receptors therefor.

A comparison of the results obtained in positions 1 and 2 shows that the butterflies actually responded less frequently in the latter than they did in the former. This comparatively small number of responses in position 2 must not be construed to mean that distilled water is without effect on the tarsi. As we shall see presently, such is not the case. It does show, however, when compared with results obtained in position 3, that distilled water is a vastly less effective stimulus for the tarsal chemoreceptors than is applejuice. The difference in the results obtained in positions 2 and 3, furthermore, demonstrates conclusively that the sense organs concerned are chemical and not tactile, for distilled water and applejuice would be indistinguishable to tangoreceptors.

In all three positions on the apparatus animals were sometimes observed to bend the antennae down to the substrate. Since pan *a*, or pan *b* was always directly beneath these organs, they were thus brought very close to, or into actual contact with, the cloth saturated with applejuice or with distilled water. There has been much evidence to show that the antennae contain olfactory organs (distance chemoreceptors). It might be argued, therefore, that in spite of the fairly uniform distribution of volatile materials over the apparatus, the ability of the animal to bring its antennae in contact with the substrates in pan *a* and pan *b* might assist materially in enabling it to distinguish between them. The marked difference between the number of responses in position 2 and that in position 3 might thus be attributed, at least in part, to sense organs on the antennae.

In order to test out the above possibility, I decided to remove the antennae and repeat the experiments. The experiments detailed in table 1 were carried out in forenoons. At the close of these experiments, the antennae of all butterflies were amputated. The labial palps and the rudimentary fore legs were likewise removed, for it seemed possible that these appendages might also possess olfactory organs. After an hour for recovery, the animals were subjected to the same number of trials as previously. One specimen became moribund during the last set of trials and had to be discarded. The total number of trials in each position was thus 23, instead of 24 as in table 1.



The results of the experiments on the mutilated butterflies are presented in table 2.

Several facts are brought out clearly by these data. In position 1 the number of responses was reduced almost to zero. It is beyond the scope of the present paper to discuss the significance of this result. It will be treated fully in a subsequent paper. In position 2 the animals behaved essentially as before the operation. In position 3 there was a considerable diminution in the number of responses. My note-book indicates that this may have been due in part to operative causes, for in several

TABLE 2  
*Pyrameis*

NUMBER OF EXPERIMENT	DURATION IN DAYS	NUMBER OF ANIMALS	POSITION 1			POSITION 2			POSITION 3		
			No response	Response		No response	Response		No response	Response	
			No extension of proboscis during 1 minute	Partial extensions of proboscis during 1 minute	Complete extension of proboscis in 1 minute or less	No extension of proboscis during 1 minute	Partial extensions of proboscis during 1 minute	Complete extension of proboscis in 1 minute or less	No extension of proboscis during 1 minute	Partial extensions of proboscis during 1 minute	Complete extension of proboscis in 1 minute or less
1	$\frac{1}{2}$	5	13	0	1	10	1	3	5	2	7
2	$\frac{1}{2}$	3	9	0	0	9	0	0	3	1	5
Totals		8	22	1		19	4		8	15	
Per cent. . .			96	4		83	17		35	65	

instances slight bleeding and subsequent coagulation caused the coil of the proboscis to become stuck together. However, the question whether the antennae, to the exclusion of the tarsi, account for the discrimination between distilled water and applejuice, is answered clearly. For, in spite of the marked diminution of responses in position 3, the animals still gave nearly four times as many responses in this position as they did in position 2. Clearly, therefore, the tarsal organs are able to distinguish between contact with distilled water and contact with applejuice, and hence, as previously contended, they must be contact chemoreceptors.

### 3. Experiments on *Vanessa*

In the experiments on *Vanessa* the procedure differed slightly from that used on *Pyrameis*. Instead of testing all animals in all three positions in each set of trials, only those were tested in position 2 which had failed to respond in position 1, and only those in position 3 which had failed to respond in position 2. The butterflies tested in position 3, therefore, were individuals which had failed to respond in either position 1 or position 2.

TABLE 3  
*Vanessa*

NUMBER OF EXPERIMENT	DURATION IN DAYS	NUMBER OF ANIMALS	POSITION 1			POSITION 2			POSITION 3		
			No response	Response		No response	Response		No response	Response	
			No extension of proboscis during 1 minute	Partial extensions of proboscis during 1 minute	Complete extension of proboscis in 1 minute or less	No extension of proboscis during 1 minute	Partial extensions of proboscis during 1 minute	Complete extension of proboscis in 1 minute or less	No extension of proboscis during 1 minute	Partial extensions of proboscis during 1 minute	Complete extension of proboscis in 1 minute or less
9	2	12	41 + 1?	7	35	40	1	1	22	5	13
11	1	8	29	2	1	27 + 1?	1	0	26	2	0
12	4	7	41 + 1?	6	39	40 + 2?	0	0	17	9	16
Totals		27 <sup>1</sup>	113	90		110	3		65	45	

<sup>1</sup> Although the total number of specimens employed appears to be 27, it was actually less, for a few animals were carried over from one experiment to another.

The trials in each set were made in immediate succession, that is, an animal failing to respond in position 1 was immediately tried in position 2, and so on. A minimal interval of one hour, however, was allowed between any two sets of trials. Three experiments were performed, the data therefrom being presented in table 3 and figure 3.

Of 203 trials in position 1, 113 gave no response; 90, response. Corresponding to the 113 failures to respond in position 1, 113 trials were made in position 2. Of these, 110 again failed to respond. The remaining three trials yielded responses, but only one of them represented a complete extension of the proboscis.

Corresponding to the 110 failures to respond in either position 1 or 2, 110 tests were made in position 3. In sixty-five of these trials the animals still gave no response, but in the remaining forty-five they responded.

In general, the results obtained on *Vanessa* were not so striking as those obtained on *Pyrameis*. This was probably due, in a large measure, to the greater proclivity for death feigning in *Vanessa*. But other factors were also concerned. Thus, in experiment 11, table 3, the results were almost uniformly negative. In fact, the animals were so unresponsive that the experiment was discontinued after the first day. When compared with

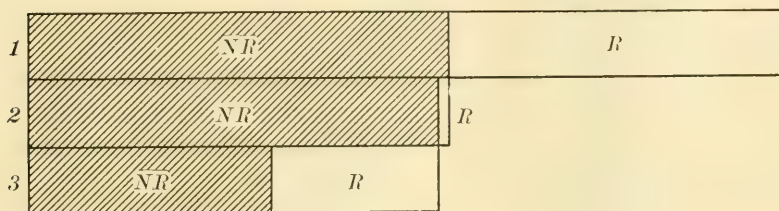


Fig. 3 Graph showing relative proportions of responses (*R*) to no responses (*NR*) obtained from specimens of *Vanessa*, when tested in positions 1, 2, and 3 on the apparatus shown in figure 1. The shaded areas represent no response; the clear areas, response. Animals which responded in position 1 were not tested in position 2, and those responding in position 2 were not tested in position 3.

experiments 9 and 12, table 3, it is clear that some general condition peculiar to experiment 11 affected the behavior of all the animals in it. I find nothing in my notes to indicate what this condition may have been, but, in the light of subsequent work, lower temperature, age of animal, and previous feeding activities may be suggested.

While the results on *Vanessa* are, in some respects, less striking, they, nevertheless, show clearly that the same conditions which obtain in *Pyrameis* also obtain in this species. Thus, there is a clear response to distance chemical stimulation. Animals failing to respond to distance stimuli, generally do the same when the second tarsi are in contact with distilled water. The contact stimulus of applejuice on the second tarsi, however,



causes a rather large proportion of these animals to respond. Vanessa like Pyrameis, therefore, must possess contact chemoreceptors in the tarsi.

#### 4. *Conclusions*

1. In Pyrameis and Vanessa the reception of distance chemical stimuli may effect an uncoiling of the proboscis.

2. Many individuals failing to exhibit the proboscis response under conditions affording only distance chemical stimuli may be induced to do so by bringing the second tarsi in contact with a substrate saturated with applejuice. There are, therefore, located in the second tarsi, receptors which are stimulated by contact with applejuice.

3. These receptors are neither touch nor temperature organs, for, as the number of responses indicates, they are differently affected by distilled water and applejuice. They must, therefore, be chemoreceptors.

4. In Pyrameis the antennae, labial palpi, and rudimentary fore legs may be removed without essentially affecting the results obtained from contact chemical stimulation of the tarsi.

#### THE PRECISE LOCATION OF THE TARSAL CHEMORECEPTORS

##### 1. *Experiments*

*a. Relation of tarsi to substrate.* In Pyrameis and Vanessa the tarsi are five-jointed. The proximal segment is the longest, being about one-half the length of the entire tarsus. It is followed by four short distal segments the terminal of which carries four claws. Normally, that portion of the leg which comes in contact with the substrate consists entirely of tarsal segments. In order to determine more precisely this relationship, a butterfly was placed in the device, as shown in figure 4, and the positions of the tarsal segments observed: first, on a hard substrate of rough white paper, and, second, on a soft substrate of absorbent cotton saturated with distilled water.

As the animal struggled, the position of the legs frequently changed. In practically all positions, however, the tibia and

the proximal segment of the tarsus formed the great shaft of the leg, running from the femur almost or quite to the substrate, while the second, third, fourth, and fifth tarsal segments, by virtue of their shortness, formed a flexible portion of the leg which was to a greater or less extent in contact with the substrate



Fig. 4 Photograph of *Pyrameis* in holder, showing position of legs on a hard substrate. *t*, tibia; *o*, proximal segment of tarsus; *s*, distal segments of tarsus. This photograph has been slightly retouched in order to bring out more clearly parts which were not quite in focus or which, because of their light color, did not show distinctly.

(fig. 4). In the great majority of cases, only the three or four distal segments were actually in contact with the substrate, and there were times when fewer than this number were affected. When all four of the distal segments were in contact with the substrate, the distal portion of the proximal segment might also be brought in contact with it. But only in rare instances, when

the animal was held very close to a substrate of wet cotton and the leg was greatly extended, was more of this segment affected. Clearly, therefore, the sense organs under consideration must be located in that part of the leg comprising the distal end of the proximal segment of the tarsus and the four distal segments of the tarsus.

*b. Local stimulation of tarsi.* In order to verify further the location of the chemoreceptors under consideration and the nature of the stimulus effective for them, the following experiment was performed. A butterfly was placed in a holder as shown in the photograph reproduced in figure 5. The substrate, however, consisted of a small screen platform mounted on wood, instead of that shown in the figure. Between the legs of one side and close to the animal's body, a pin was stuck into the wood to limit the range of the leg movement as much as possible. Local stimulation was then applied by means of a needle bearing on its end a small cotton swab, 1 cm. long and 0.1 cm. in diameter, soaked in applejuice.

The swab was first held 1 mm. anterior to the proboscis. If the animal failed to respond in one minute, as it always did, the swab was shifted to a position 1 mm. from the ventral surface of one of the tarsi. Here it was also held for one minute or until a response occurred. If no response occurred in one minute, the swab was placed in contact with the tarsus. I endeavored to maintain the swab in continuous contact with the tarsus and to confine the contact to the four distal segments. Movements of the leg, however, frequently prevented the realization of these conditions. Thus, contact might be lost for an instant or the proximal segment might make contact with the swab. But the tibia was kept free from any contact stimulation.

Three butterflies were experimented upon, and, with one exception, two trials were made on each of the four ambulatory tarsi of each specimen. The total number of trials for each position of the swab was thus twenty-three or twenty-four. The butterflies were the same specimens that had served in experiment 2, table 1, and experiment 2, table 2, in the course of which the antennae, labial palpi, and rudimentary fore legs



had been amputated. It should be borne in mind, therefore, that these were operated specimens. The results of these experiments are presented in table 4.

It will be noted that there was not a single response when the swab was held 1 mm. anterior to the proboscis. Moreover,

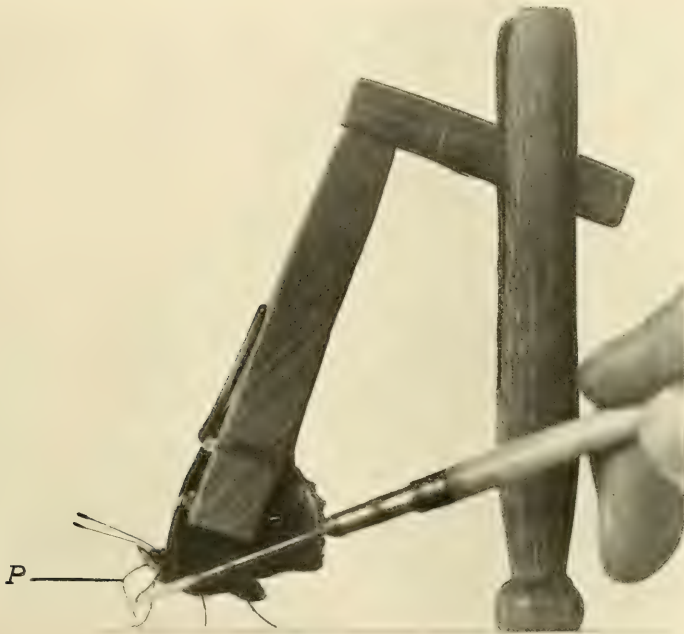


Fig. 5 Photograph of the same specimen of *Pyrameis* as shown in figure 4, showing the proboscis, *p*, completely extended as a result of contact between the left second tarsus, and a cotton swab saturated with a 1 M saccharose solution. This photograph has been slightly retouched in order to bring out more clearly parts which were not quite in focus or which, because of their light color, did not show distinctly.

there was but one response when the swab was held 1 mm. from the ventral surface of the tarsus. When, however, the swab was applied directly to the ventral surface of the tarsus, there was not a single failure to respond, and in twenty of the twenty-three trials the proboscis was completely extended.

These responses are illustrated by two photographs of normal animals reproduced in figures 5 and 6.

As stated above, one butterfly responded in one instance when the swab was held 1 mm. from the ventral surface of the tarsus. This was a perfectly clear response, the proboscis being uncoiled fully one-half. I am not prepared to explain the case with certainty, although the following excerpt from my notebook is suggestive: "Right hind foot (the one being tested) moved. Fore foot (anterior ambulatory) may have touched

TABLE 4  
*Pyrameis*

COTTON SWAB 1 CM. X 0.1 CM., SOAKED IN APPLEJUICE	NO RESPONSE	RESPONSE	
	No extension	Partial extensions	Complete extension
1 mm. anterior to proboscis.....	23	0	0
1 mm. from ventral surface of left second tarsus..	6	0	0
1 mm. from ventral surface of right second tarsus..	6	0	0
1 mm. from ventral surface of left third tarsus....	6	0	0
1 mm. from ventral surface of right third tarsus...	5	1	0
In contact with ventral surface of left second tarsus.....	0	1	5
In contact with ventral surface of right second tarsus.....	0	0	6
In contact with ventral surface of left third tarsus	0	2	4
In contact with ventral surface of right third tarsus.....	0	0	5

swab, though I think not." Whatever the correct explanation may be, the important result of this experiment is not, that in one instance a butterfly responded, apparently without contact between swab and tarsus, but rather that in all other cases the animals failed to respond until such contact had been made. It is clear, therefore, that the organs under consideration are *contact* chemoreceptors, and not distance chemoreceptors.

I have made a few trials with local stimulation on *Vanessa*, but the animals failed to respond in practically every case. While the data are too few to warrant any conclusion, it is my belief that here again the death-feigning instinct was largely responsible for the results obtained.

## 2. Conclusions

1. While the exact location of the tarsal chemoreceptors is not yet determined, it is clear, from a study of the normal



Fig. 6 Photograph of the same specimen of *Pyrameis* as shown in figures 4 and 5, showing the proboscis, *p*, completely extended as a result of contact between the left hind tarsus and a cotton swab saturated with a 1 M saccharose solution. The detail of the photograph is not sufficient to bring out the left hind leg, but the other three walking legs are clearly visible in contact with the substrate. This photograph has been slightly retouched in order to bring out more clearly parts which were not quite in focus or which, because of their light color, did not show distinctly.

position of the leg, that these organs must be present in that part of the tarsus constituted by the distal end of the proximal segment and the four distal segments.

2. The conclusion stated above, viz., that in *Pyrameis*, the removal of the antennae, the labial palpi, and the rudimentary



fore legs, with whatever chemoreceptors they may possess, in nowise affects the response evoked by contact chemical stimulation of the tarsi, is further confirmed.

3. The tarsal chemoreceptors are stimulated through intimate contact with the source of stimulating materials, and are, therefore, contact chemoreceptors.

4. The receptors under consideration are, at least in the case of *Pyrameis*, present in the tarsi of all four of the walking legs.

#### SOME PRELIMINARY WORK ON THE NATURE OF THE TARSAL CHEMORECEPTORS

##### 1. *Experiments*

Having located the chemoreceptors of the four tarsi, I was interested to discover, if possible, what substances the animal was able to discriminate through these organs. There can be no doubt that *Pyrameis*, as well as *Vanessa*, is able to distinguish certain substances from others in this way. The data presented in tables 1, 2, and 3 show unmistakably that both species distinguish applejuice from distilled water. The question, therefore, to be answered was what classes of substances might be distinguished. In man, there are four primary sensations of taste, viz., sweet, sour, bitter, and salt. Pure water is to us tasteless, although we readily detect its presence on the tongue through touch and temperature organs. There is no reason to suppose, a priori, that the same conditions necessarily hold for *Pyrameis*. Substances which we taste may not be effective for the tarsal organs of the butterfly, and vice versa. The data presented in this connection are the results of preliminary experiments only, but I have felt it advisable to present them here, even though their final significance must be judged, at least in part, in the light of further work. This work is now under way.

The method of experimentation was as follows. A Syracuse watch-glass was filled with a thin, uniform pad of absorbent cotton, cut to fit the glass, and the cotton was thoroughly saturated with the solution to be tested. The butterfly to be tested

was placed in a holder and held on clean filter-paper for thirty seconds to overcome any death feigning and to make certain that the proboscis exhibited no sign of movement. The animal was then transferred to the center of the watch-glass, where it was held with all four walking legs in contact with the cotton pad. There was enough solution present to immerse the distal ends of the tarsi, if the legs were pressed firmly against the cotton. In struggling, the butterfly frequently did this, and if it failed to do so, a little pressure on the holder easily brought about the same result. This manipulation, however, was seldom necessary, as mere contact with the cotton was generally sufficient to produce a response if any was given at all. As in previous experiments, a failure to observe any visible extension of the proboscis within a period of one minute was considered a 'no response.' Immediately after each trial the feet of the butterfly were immersed in distilled water and thoroughly washed, in order to remove any adhering material which might contaminate succeeding trials. A minimum interval of fifteen minutes was allowed between the trials of a single individual.

Four solutions and distilled water were tested in the above manner. The solutions were 1 M saccharose, 1 M hydrochloric acid, 1 M sodium chloride, and M/600 quinine sulphate. In the introductory paragraph of this paper, especial stress was laid upon the importance of using stimuli which the animal encounters in its normal environment, and for this reason apple-juice was employed in a number of experiments. Some of the substances mentioned above are rarely or never met with by butterflies in their natural state. It was necessary to select these substances, however, because their solutions best met the requirements of the experiment, viz., to afford widely different contact stimuli along with uniform distance stimuli.

It seemed possible by repeated trials on each of a large number of butterflies, that the number of responses to each of the various substances employed might be taken as a measure of its effectiveness, and by comparison of these data any discrimination might be demonstrated. Three experiments were performed, involving a total of twenty-four butterflies, all of which had been reared

in captivity. Eighteen of the animals were 51 to 75 hours old; six, 50 to 98 hours old. None had received either food or water after emerging from the pupae. Inanition greatly increased the responsiveness of the animals, particularly to water, as tests at various ages clearly demonstrated. During the first forty-eight hours after hatching butterflies never responded either to distance or to contact stimulation of water. After this period, however, they began to respond more freely, and the number of responses rose rapidly to nearly 100 per cent, remaining there

TABLE 5  
*Pyrameis*

NUMBER OF EXPERIMENT	NUMBER OF ANIMALS	PERIOD OF INANITION	1 M SACCHAROSE			DISTILLED H <sub>2</sub> O			1 M HCl			M/600 QUININE SULPHATE			1 M NaCl		
			No re- sponse		Re- sponse	No re- sponse		Re- sponse	No re- sponse		Re- sponse	No re- sponse		Re- sponse	No re- sponse		Re- sponse
			No extension	Partial extensions		No extension	Partial extensions		No extension	Partial extensions		No extension	Partial extensions		No extension	Partial extensions	
				Complete extension	Complete extension		Complete extension	Complete extension		Complete extension	Complete extension		Complete extension	Complete extension		Complete extension	Complete extension
		hours															
1f	6	51-75	0	0	12	2	1	9	4	2	6	6	1	5	8	1	3
2f	12	51-75	0	0	24	4	3	17	9	3	12	9	3	12	11	3	10
3f	6	50-98	0	0	12	1	1	10	1	2	9	2	0	10	3 + 1?	2	6
Totals.....			0	48		7	41		14	34		17	31		23	25	
Per cent....			0	100		15	85		29	71		35	65		48	52	

until the animals became moribund. Starvation, therefore, renders the animals more responsive to water, and essentially the same condition undoubtedly holds for food substances also. The data from these experiments are presented in table 5.

The results in the different experiments are very uniform and bring out several facts clearly. First, each of the four solutions employed may effect the proboscis response. Second, distilled water alone is a very effective stimulus for the tarsi, being second only to 1 M saccharose. Third, while all of the five substances employed are able to effect the proboscis response, they are not equal in this respect. The order of their effectiveness, based on



the number of responses obtained from a group of animals considered collectively, is, 1 M saccharose > distilled water > 1 M hydrochloric acid > M/600 quinine sulphate > 1 M sodium chloride.

The effectiveness of pure water in stimulating the tarsi in butterflies is of particular interest in connection with the habit of many species to gather in moist places and suck up water. The cloud of butterflies about the drying mud puddle in summer is familiar enough. Tutt ('97) has collected a number of exceedingly interesting observations on the drinking habits of butterflies and moths. Sometimes the amount of water consumed by these animals is almost inconceivable. Thus, Tutt ('97, p. 77-78) quotes Baron as follows:

One morning, whilst sitting by the side of one of these streams, I noticed the *Papilio*, which is an insect measuring four inches across the wings, resting on a wet bank, and, wishing to procure it as a specimen, I approached it as gently as possible, the creature being apparently so absorbed in what it was about as to be totally unconscious of my proximity to it. Noticing strange and unaccountable movements—sundry jerks and probings with its proboscis—I quietly sat down near it, in order to watch it more closely. I observed that every second or two a drop of pure liquid was squirted (not exuded merely) from the tip of its abdomen. I picked up a leaf that was lying near, and inserted the edge of it between the insect's body and the ground, so as to catch the liquid. Unfortunately I had no watch with me at the time nor means of measuring liquids, but I reckoned that about thirty drops were emitted per minute. I held the leaf for about five minutes, as nearly as I could reckon, and at the end of that time there was caught in it about a saltspoon of what seemed to be pure water, without either taste or color. After watching the butterfly for a time I seized it by the wings between my thumb and fingers with the greatest ease, so utterly lost did it appear to be to what was going on near it. In another spot I saw as many as sixteen of these large butterflies within the space of a square foot, all engaged in the same strange action. Some of them emitted the liquid more frequently than others; and one of them squirted the liquid so as to drop fully a third of an inch beyond the point on the ground perpendicular with the end of its body. It was at this spot that I saw the second species of butterfly alluded to, *Appias saba*, also engaged in the same curious proceeding.

Of course, caution is necessary in interpreting observations such as the above, for dissolved substances in the water undoubtedly play a considerable rôle. The responsiveness to water,

alone, however, may also be the correct explanation of many such instances, and, in view of the sensitivity of the tarsi, it is not difficult to understand why the butterflies remain for long periods of time with proboscis extended, either probing the ground or sucking up the water.

A very natural question suggests itself in connection with the effect of distilled water on the tarsi. Does water stimulate the same receptors that are affected by the various solutions employed, or does it, as in man, merely afford tactile and temperature stimuli to which the butterfly responds. I have no data with which to answer this question. The simpler hypothesis, in the absence of evidence, is that a single type of sense organ is here operative, and it is my belief that this is correct.

As stated above, the difference in the number of responses to the various substances employed indicates that the butterflies clearly discriminate between at least some of these substances. Under the conditions of the experiment, a 1 M solution of saccharose was 15 per cent more effective than distilled water, while solutions of 1 M hydrochloric acid, M/600 quinine sulphate, and 1 M sodium chloride were from 14 to 33 per cent less effective than distilled water. There can be no doubt, therefore, that the tarsal chemoreceptors are able to distinguish a 1 M solution of cane-sugar from distilled water or a 1 M solution of sodium chloride from either of these. It is interesting also to note that the order, 1 M saccharose > distilled water > 1 M hydrochloric acid > M/600 quinine sulphate > 1 M sodium chloride, is not the order of osmotic effectiveness for these substances, so that the tarsal receptors cannot be osmotic organs, but must be considered as true chemoreceptors.

We have already seen that the removal of antennae, labial palps, and rudimentary fore legs does not materially affect the response evoked by contact chemical stimulation of the tarsi. It has also been demonstrated that *Pyrameis* is able to distinguish between various substances through the tarsal organs. Therefore, the removal of the antennae, labial palps, and rudimentary fore legs should not exert any important effect on the degree of responsiveness to different chemical stimuli. This

was tested. At the close of the last two experiments described above (table 5, 2f, 3f), the animals were fed on 1 M saccharose solution. In experiment 2f the twelve butterflies, after being fed, were 'rested' for forty-eight hours. The antennae, labial palpi, and rudimentary fore legs were then removed. Seventeen hours after the operation, sixty-five hours after being fed, the nine surviving animals were subjected to the same experiment as before. In experiment 3f a similar procedure was followed, the animals being 'rested' forty-three hours after feeding, operated upon, and one hour later subjected to experiment.

TABLE 6

*Pyrameis*

NUMBER OF EXPERIMENT	NUMBER OF ANIMALS	PERIOD OF INANITION	1 M SACCHAROSE			DISTILLED H <sub>2</sub> O			1 M HCl			M/600 QUININE SULPHATE			1 M NaCl		
			No re- sponse		Re- sponse	No re- sponse		Re- sponse	No re- sponse		Re- sponse	No re- sponse		Re- sponse	No re- sponse		Re- sponse
			No extension	Partial extensions		No extension	Partial extensions		No extension	Partial extensions		No extension	Partial extensions		No extension	Partial extensions	
		hours		Complete	extension		Complete	extension		Complete	extension		Complete	extension		Complete	extension
2g	9	65	1	0	8	1	0	8	0	0	9	1	0	8	1	2	6
3g	6	44	0	0	12	4	1	7	7	0	5	7	1	4	0	0	12
Totals.....			1	20		5	16		7	14		8	13		1	20	
Per cent.....			5	95		24	76		33	67		38	62		5	95	

The results obtained from the operated animals are presented in table 6. They agree in every respect but one with the results obtained from unoperated animals. The one point of difference is in experiment 3g, in which a 1 M NaCl solution, instead of being the least effective of the substances employed, became equal to the most effective and produced a response in every trial. The loss of sense organs entailed by the removal of the appendages enumerated cannot account for this result. For, under the conditions of the experiment, such sense organs could have been



affected only by distance stimuli, and these were identical for all the substances employed, viz., water vapor. Other explanations must, therefore, be sought.

Let us consider this difference in responsiveness to sodium chloride more fully. The results of tables 5 and 6 are based on too large a number of observations for us to suppose that either nullifies the significance of the other. The result in each table undoubtedly gives a correct idea of the responsiveness of animals under those particular conditions. The only possible conclusion, therefore, seems to be that the responsiveness to a 1 M solution of sodium chloride may vary from time to time with the physiological state of the animals. This explanation becomes more plausible when the data from which tables 5 and 6 are compiled are analyzed with respect to individual specimens instead of the entire group. It then appears that while a few butterflies may vary in the course of a single experiment in their responses to sodium chloride, the great majority are constant, either responding or failing to respond, alike in every trial. The correct explanation, therefore, seems to be that in experiments 2f and 3f (table 5) there were a number of butterflies which were unresponsive to NaCl, while in experiments 2g and 3g, due to some physiological change, a number of these individuals had become responsive. Besides this normal variability, possible effects of the operation must not be overlooked. In practically all animals there was a loss of blood. It is true this was very slight in most cases. Nevertheless, it is entirely conceivable that a factor of this sort might considerably increase the sensitivity to a salt solution.

In experiment 2g, table 6, and, to a less extent, experiment 3f, table 5, the number of responses was about the same for each of the five substances. In both of these experiments the period of inanition undergone by the animals was greater than was the case with the other experiments of the same series. Thus, in experiment 3f the animals were 50 to 98 hours old, while in experiments 1f and 2f they were but 51 to 75 hours old. The specimens in 3f, therefore, may have been as much as twenty-three hours older than specimens in 1f and 2f. In the case of

experiment 2g, a similar situation held. The butterflies of this experiment were tested after sixty-five hours without food or water, while in experiment 3g, the other experiment of the series, they had been but forty-four hours without these. There was, therefore, a difference of twenty-one hours of inanition in this case also. The increased period of inanition together with the great sensitivity of the tarsal receptors to water may perhaps account in part for this uniformity of response to different solutions and to distilled water. As previously pointed out, butterflies were found to become more and more responsive to the stimulation of water, the longer the period of inanition was prolonged. It is possible, therefore, that in the cases under discussion, the response to water had become so strong that the differential effects of the various solutes were somewhat obscured.

In conclusion a word may be said concerning the uses of the tarsal chemoreceptors. From what has been shown in the present paper, it is clear that one very important function, perhaps the sole function, is in connection with food substances and water. Not only does the butterfly detect the presence and exact location of such substances through these organs, but it also discriminates to some extent the nature of the substance. Whether this discrimination is final or merely preliminary cannot be stated at present. It would be interesting indeed to know whether the proboscis, once it has been extended through stimulation of the tarsal organs, ever refuses the intake of the stimulating substance. This point I hope to settle in the course of further work. Whatever other organs may assist in the final discrimination of food substances, it is certain that they are first passed upon by the tarsal chemoreceptors. These organs, therefore, function somewhat as organs of taste, and *Pyrameis* and *Vanessa* may be said to taste with their feet.

## 2. Conclusions

1. In *Pyrameis* the degree of responsiveness to contact chemical stimulation of the tarsi is different for different substances.
2. By means of the tarsal organs, *Pyrameis* is able to distinguish a 1 M saccharose solution from distilled water, and

such solutions as 1 M HCl, M/600 quinine sulphate, and 1 M NaCl from either 1 M saccharose or distilled water.

3. Distilled water alone is a very effective stimulus when applied to the tarsi.

4. The responsiveness to a 1 M NaCl solution may vary widely from time to time.

5. The ability of *Pyrameis* to distinguish the chemical nature of substances in contact with its tarsi is independent of whatever sense organs may be present in the antennae, labial palpi, and rudimentary fore legs.

6. The tarsal organs are not osmotic organs.

#### GENERAL SUMMARY AND CONCLUSIONS

1. *Pyrameis atalanta* Linn. and *Vanessa antiopa* Linn. may respond to the distance chemical stimuli afforded by applejuice by uncoiling the proboscis.

2. Many individuals failing to respond to these distance stimuli alone, may be made to respond by bringing the second tarsi in contact with the applejuice. If contact with distilled water be substituted for contact with applejuice, the number of responses is greatly reduced. Contact between the tarsi and certain substances is, therefore, also able to effect the proboscis response.

3. The effectiveness of a given substance in stimulating the tarsi is indicated by the percentage of responses which it produces. In this manner it can be shown that the sense organs concerned are neither temperature organs, tactile organs, nor osmotic organs, but chemical organs.

4. The tarsal chemoreceptors are stimulated only through intimate contact with the stimulating material, and hence are contact chemoreceptors.

5. The tarsal chemoreceptors are present, in the case of *Pyrameis*—and the same probably holds for *Vanessa* also—in all four tarsi of the walking legs. They are located in that portion of the tarsi comprised by the distal end of the proximal segment and the four distal segments.



6. *Pyrameis* and *Vanessa* are able to discriminate through their tarsi between applejuice and distilled water. *Pyrameis* is also able to distinguish a 1 M saccharose solution from distilled water and such solutions as 1 M HCl, M/600 quinine sulphate, and 1 M NaCl from either distilled water or 1 M saccharose.

7. Distilled water itself, when applied to the tarsi, is a most effective stimulus in evoking the proboscis response.

8. The responsiveness to stimulation of the tarsi by a given solution, such as 1 M NaCl, for example, may vary widely from one day to another. This variability is undoubtedly conditioned by changing physiological states in the animals.

9. The removal of the antennae, labial palpi, and rudimentary fore legs in *Pyrameis* does not affect in any significant way the responses produced through contact chemical stimulation of the tarsi.

10. One very important rôle of the tarsal chemoreceptors, perhaps their only rôle, is in connection with food substances and water. Not only is the animal able to detect the presence and exact location of such substances by means of these organs, but it is also able to make some discrimination as to their nature. The tarsal organs, therefore, appear to function somewhat as organs of taste.

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Resumen por el autor, George H. Parker,  
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La locomoción de la holoturia *Stichopus panimensis* Clark.

*Stichopus panimensis* se arrastra sobre su trivium por medio de ondas locomotrices de un tipo monotáxico directo. Estas ondas progresan sobre el animal a una velocidad de unos 0.39 cm. por segundo, y le permiten andar un metro en unos quince minutos. La parte del trivium que se mueve hacia delante se levanta marcadamente sobre el substrato, al cual está aplicado el resto del trivium mediante los pies ambulacrales.

Translation by José F. Nonidez  
Cornell Medical College, New York

## THE LOCOMOTION OF THE HOLOTHURIAN *STICHOPUS PANIMENSIS* CLARK<sup>1</sup>

G. H. PARKER

ONE FIGURE

*Stichopus panimensis* is a holothurian found in considerable numbers on the rocky shores near the Scripps Institution for Biological Research at La Jolla in southern California. Living individuals under normal conditions measure about 25 centimeters in length. In relatively quiet waters they may be seen creeping about in a manner that resembles superficially the locomotion of a gigantic caterpillar, in that waves of movement pass over them from end to end.

*Stichopus* normally attaches itself to the substrate by its trivium, whose three rows of ambulacral feet are essential to its locomotion. Creeping is accomplished in part by a muscular wave that originates at the posterior end of the animal and sweeps over it to the anterior end. Before the locomotor wave begins the whole length of the body of *Stichopus* is attached to the substrate by its numerous ambulacral feet (fig. 1, *A*). With the first appearance of this wave, the feet of the posterior portion are loosened from the substrate and the whole hind end is lifted well above that surface. The posterior portion of the animal then contracts vigorously on its length thus carrying the hind end forward to a new position (*B*), in advance of that which it formerly occupied.

In this new position the posterior portion is then reapplied to the substrate, to which its ambulacral feet again become attached while the wave moves on to the middle of the animal (*C*). As this portion is becoming attached, the wave reaches the head, which is now projected forward (*D*) and finally attached to the substrate, when the condition characteristic of rest is

<sup>1</sup> Contributions from the Zoölogical Laboratory of the Museum of Comparative Zoölogy at Harvard College, No. 329.



resumed (*E*). The effect of the locomotor wave as it passes off at the anterior end of the animal is to carry this end as far forward as the posterior end was advanced, and in this way *Stichopus* moves forward step by step over the substrate.

Animals about 25 centimeters in length were found to creep a meter in about 15 minutes. When they were watched closely, they were found to draw the tail forward and project the head about 7 centimeters for each locomotor wave and these waves occurred about once a minute. Never more than one wave at a time was observed on the body of *Stichopus*. Usually a few

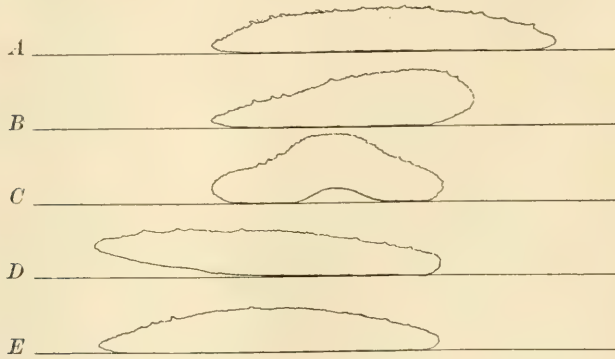


Fig. 1 Diagrammatic figures of the passage of a single locomotor wave over the body of a *Stichopus panimensis*. *A*, resting position; *B*, initiation of wave at posterior end; *C*, wave at middle of body, posterior end returned to substrate; *D*, wave passing off at anterior end; *E*, new resting position.

seconds intervene between the time at which a given locomotor wave disappears from the anterior end and a new one appears at the posterior end.

In an individual whose resting length was between 24 and 25 centimeters and whose creeping was watched for some time, the locomotor waves passed over its body in intervals that varied between 52 and 70 seconds with an average of 63 seconds. Consequently the rate of these waves must have been about 0.39 centimeters per second, or approximately one-sixth to one-seventh as rapid as that of the neuromuscular wave of a creeping earthworm.

As each locomotor wave in *Stichopus* represents a region of freedom from the substrate to which the remainder of the trivium is temporarily attached, and as each wave progresses from the hind end of the animal to the head, the locomotion of this echinoderm reproduces the essentials of that type of gastropod locomotion which has been designated the direct monotaxic type (Vlès, '07). This type is well shown in *Helix* (Parker, '11), though for comparison with *Stichopus* certain sea-anemones (Parker, '17 a) are better, for in these forms, as in *Stichopus*, only one wave at a time occurs on the foot as contrasted with the numerous waves seen in *Helix*. For the analysis of this type of locomotion *Stichopus* is a particularly satisfactory form to study because of the grossness of its movements. In this holothurian there can be no question that in the region of the locomotor wave the foot is lifted well above the substrate, a point that cannot always be easily seen in gastropods. And, further, in *Stichopus* the means by which the foot is attached to the substrate does not have to be inferred as in the case of some gastropods, but is to be directly observed in the ambulacral feet of the holothurian. In both these respects *Stichopus* is a more favorable animal for the analysis of this general type of locomotion than even *Aplysia* (Parker, '17 b), in which the temporary elevation of the foot and its subsequent attachment to the substrate can be seen with unusual clearness.

#### SUMMARY

*Stichopus panimensis* creeps on its trivium by means of locomotor waves of a direct monotaxic type.

These waves progress over the animal at a rate of about 0.39 centimeters per second and enable the animal to creep a meter in about 15 minutes.

The part of the trivium moved forward is lifted well above the substrate to which the remainder of the trivium is attached by the ambulacral feet.

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Resumen por el autor, E. C. MacDowell,  
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El alcoholismo y el comportamiento de las ratas blancas.  
I. La influencia de los abuelos alcohólicos sobre el comportamiento de los individuos en un laberinto.

El presente trabajo trata del comportamiento de los individuos de la segunda generación no alcohólica (procedentes de una generación a la cual fué suministrado alcohol por el método de la inhalación), cuando se les coloca en un laberinto para que busquen la salida. Los individuos escojidos como tipo de comparación fueron los nietos de los hermanos y hermanas normales de los animales tratados por el alcohol. En el curso de todos los experimentos se cruzaron hermanos con hermanas.

El tratamiento mediante el alcohol no produjo anormalidades estructurales marcadas en la generación de los nietos; pero pareció modificar el comportamiento. En esta generación el autor enseñó a 31 ratas, procedentes de los individuos alcohólicos, y 29 ratas normales a buscar la salida en un laberinto circular de Watson, mediante tres pruebas diarias durante un periodo de doce días. Cuando se comparan los individuos de ambas clases, tomando como criterio el tiempo empleado, la distancia recorrida, los errores hechos, el número de pruebas perfectas, la ocurrencia de las primeras pruebas perfectas y el tiempo empleado en ellas, puede comprobarse que los nietos de los alcohólicos, tomados en conjunto, son más torpes que los normales en aprender la salida al centro del laberinto.

Translation by José F. Nonidez  
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# ALCOHOLISM AND THE BEHAVIOR OF WHITE RATS

## I. THE INFLUENCE OF ALCOHOLIC GRANDPARENTS UPON MAZE-BEHAVIOR<sup>1</sup>

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SEVENTEEN FIGURES

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<sup>1</sup> This is one of a series of papers on the general subject. Following studies will cover the results of training these same rats upon a multiple-choice apparatus, and of training the parental and grandparental generations.



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## INTRODUCTION

The following experiments have been carried on primarily to try to control or modify inheritance. It must be made clear in the beginning that whatever human relation they have is incidental. The human interest is more especially aroused by the chemical used than by any parallelism of the kinesthetic behavior of rats in a maze and the moral behavior of man. On the other hand, if it can be proved that alcohol does occasion changes in the normal inheritance of rats, as shown even by such an elementary type of behavior as learning a maze, a great field of possibilities is at once opened up; other modifications of inheritance, by means of the same or other chemicals, may be brought about in other animals, including man.

The general plan of the work undertaken has been to study the habit-forming abilities of rats in successive inbred generations following one generation treated with alcohol. For a standard or control the inbred descendants of the normal brothers and sisters of the alcoholized rats have been used. Preliminary studies indicated that strong doses would be required if any influence upon the germ-plasm is to be expected. For this reason the maximum exposure to alcohol fumes has been employed in the treatment of the grandparents of the rats whose training is the subject of the present paper. The results appear to indicate that inheritance can be modified to a certain degree by alcohol.

## METHODS

*a. Source of material*

The rats used in this paper were raised from a series of three brother-by-sister matings; two of these matings were made from two litters of rats from The Wistar Institute Standard Stock. The third line resulted from the combination of four lines from independent sources, including The Wistar Institute and Johns Hopkins University; these were brought together in a series of experiments on the same subject in which all the matings were made between unrelated individuals, instead of between brothers and sisters, as in the present series. The pedigrees of the rats are given in figure 1; those given alcohol were marked 'A.' In each strain one pair was given the alcohol treatment and one pair, litter mates of the alcoholized rats, was raised as normal controls. When the alcohol treatment was started, the plans involved a very much larger number of rats than appears in the results. The present report is based on the alcoholization of six rats, although 112 rats were given the alcohol treatment. The great reduction in the size of the experiment was due to circumstances resulting from the war. In the third strain there appears a second pair of rats (101/125) marked 'A.' These were concerned in the first series of experiments and were given a light dosage, increasing from thirty minutes a day at thirty days up to ninety minutes a day at fifty days. Since these rats are the common ancestors of both the test and the control animals in this strain, it is clear that the control rats in this strain are not strictly comparable with those in other strains; but within this strain the controls are strictly comparable with the alcoholized rats. Moreover, the three strains may still be put together, because: 1) the results (unpublished) of training the offspring of the lightly alcoholized ancestors, that is, the generation to which the pair 295/286 belongs, appear to indicate that the treatment of the parents did not modify the behavior of the offspring; 2) even if there should remain some effect of the first alcoholization, this would work in the direction of reducing the difference that has been found between the tests and controls;

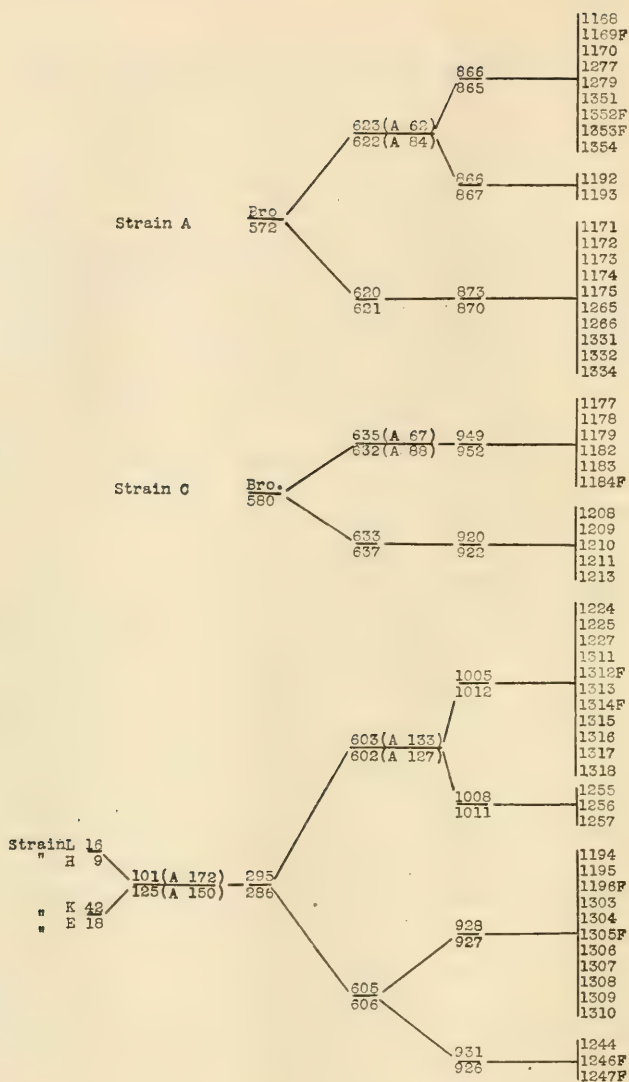


Fig. 1 Pedigrees of the rats used. Parents shown in the form of fractions; males above the line, females, below. 'Bro.' indicates that the father was a brother of the mother. Within each strain all the rats in the column at the right (those discussed herein) came from a single pair of great-grandparents, which were themselves brother and sister. Rats marked (F) are called 'failures,' meaning that they had one or more incomplete trials; those marked 'A' were treated with alcohol; the numbers in parentheses tell how many days of the rats' treatment could have influenced the offspring. One pair of rats in each strain were given alcohol till they were dead drunk once a day; pair 101/125 in strain L was given a light dose, measured by the time of exposure (ninety minutes) which appeared to cause no intoxication.

3) since there are approximately equal numbers of tests and controls in this strain the comparative result is left unchanged. The term 'test' is used for a rat receiving alcohol and for its descendants. In the present paper it will refer to the grandchildren of alcoholized rats.

*b. Alcoholization of the grandparents*

When the rats to be alcoholized were four weeks old they were weaned; half the litter was saved as control and the other half started with the alcohol treatment. This division of the litter was made purely at random, obviously with no knowledge of the animals' behavior tendencies. Differences in weight at this age are very small and do not bear a very high correlation with adult size. Only litters with at least an even number of males and females could be used; odd individuals were held as stock.

The treatment was started by placing the rats in tanks of alcohol vapor for thirty minutes a day. After a week of this, the rats were left in the tanks until they were obviously influenced by the alcohol; that is, until they showed some lack of control of motion. This would require from one to two hours. This treatment was given for a week. Then they were left in the tanks until they were dead drunk and lay limp; this took from two to three hours for the young rats and from three to four hours for the old ones. The females received this treatment each day, even through the period of gestation up to a day or two before the birth of the litter, and it was resumed after the young were weaned at twenty-eight days; for the males the treatment was given every day. In this way the effective dosage of the two parents was different and it increased for successive litters. The dosage affecting each of the test rats is indicated in the pedigree chart. The numbers in parenthesis after each grandparent represent their respective doses, that of the male, the number of days before conception, and of the female, the number of days up to the day of the birth of the litter.

As far as possible, the treatment of the rats in the first generation was uniform, with the exception of the alcoholization. The



normal rats in a litter were raised in cages directly above the cages of their respective brothers and sisters that were being alcoholized, in order to equalize the possible influence of the immediate surroundings. Before maturity the rats were separated into pairs; from each litter an equal number of alcoholic and normal pairs were mated. No alcohol was given to the rats in the subsequent generations. The largest rats among the offspring were chosen as parents of the next generation, with no knowledge of their training records. As many rats as possible from these matings of the progeny of alcoholized rats were to have been raised, but here again the experiment was unavoidably much reduced.

*c. The alcohol tanks*

Tanks of galvanized iron, 30 by 16 by 12 inches, with tight-fitting covers, held the alcohol fumes and the rats during treatment. Inside were false bottoms of wire netting which held the rats above the alcohol filling the bottoms of the tanks. The covers were made with glass windows so the condition of the animals could be observed. A small hole in each end, at the level of the wire bottom, was made, on the supposition that the carbon dioxide might thereby tend to escape. In an earlier series of experiments the controls were daily placed in tanks of the same description without alcohol, as long as the test rats were in their tanks. Merely the confinement in the limited air space caused none of the symptoms observed in the rats from the alcohol tanks. The alcoholized rats would frequently remain unconscious for hours after they were returned to their cages; the controls upon leaving the control tanks would show no modification of their normal behavior. It can safely be concluded that the apparent intoxication was due to the alcohol fumes, and not to the confinement in tightly closed tanks.

*d. Training; apparatus and methods*

The apparatus used in training these rats was constructed after the plan of Watson ('14), namely, a circular maze con-

sisting of five concentric alleys with doorways and blind alleys so arranged that the correct path from the outside to the center required a rat to turn alternately to the right and then to the left at the successive doorways. Two large mirrors were suspended from the ceiling above the maze in such a way as to direct the image of the maze into a set of lenses that focused it, greatly reduced, upon a sheet of paper. With the maze brilliantly illuminated and the light cut off from the paper by a dark box, the observer could plainly see on the paper the image of the rat as it went through the maze and could make a permanent record of the rat's course by following it with a pencil. A diagram of the maze as it appeared reflected on the paper was printed upon the sheets used to record the course of every trial of each rat (fig. 7). Frequently it was necessary to use several sheets to record a very long trial, in order to make it possible afterward to understand the course taken by the rat. The time required for each trial was recorded on these sheets.

When the rats were forty-nine days old their training was started. They were given seven days of preliminary training, which consisted of feeding them in the inner circle of the maze shut off from the alley. This was followed by the training proper (subsequently to be called 'training'), consisting of three successive trials a day for eight days. The rat was allowed to taste the food between trials, but was removed from the center as soon as it had taken a bite; after the third trial the rat was allowed to eat for five minutes. The food consisted of white bread and milk. At the end of the eight days of training, the rat was started upon the preliminary training on a multiple-choice apparatus. Thirty-one days were spent on the multiple-choice apparatus. At the end of this time the rat was returned to the maze to test its retention of the maze habit. The retention trials were continued, three a day, for four days. The results of the training on the multiple-choice apparatus will appear in a later paper.

*e. 'Failures,' 'incompletes' and 'completes'*

If the rat did not happen to reach the center in a reasonable time on the very first trial, it was removed, fed in the center, and tried again the next day. In such cases all the time spent on different days in reaching the center for the first time was counted as the first trial. In certain cases (those rats marked 'F' in the pedigree chart) after a rat had made one or more successful trials and then failed to reach the center after from ten to twenty minutes, the rat was called a 'failure' rat, or just a 'failure.' The time given before such trials were called off was not always the same, for in some trials the rat would continue to be active and keep investigating the alleys, in other cases the rat would give up the problem and lie still. When the rat did this, it was found useless to wait longer, while it seemed wiser to let a rat continue its searching if it was active, and especially if it was in an inner alley. During the experiment the difficulty of handling the data of rats with such failure records was not fully realized, or else a different plan would have been followed. As it was, the rats with these failures were continued throughout the training (in most cases the rat succeeded on the day following a failure); at the end of their training they gave about as good records as the rats in the same group that did not have failures. However, the influence of such failures on the following days remained a serious question. The trials immediately following are slower than the average of the other rats in the same group; even though this difference seems to disappear in later trials, the effect may none the less be operating. This makes the treatment of the data from such rats a matter of difficulty. The time spent on an unsuccessful trial could be added to the following successful trial as was the practice when a rat did not succeed on the first trial. But failing after the center has once been reached is an entirely different matter from not reaching the center for the first time. These failures are not of frequent occurrence; they never occur after the third day, and most of them occur on the first day; only ten rats out of sixty had a 'failure' and only one rat failed a second time after another successful trial. It seems as though some unusual



temporary circumstances have occasioned this unusual behavior. That just these rats and not others responded in this manner may have been due to some particular sensitiveness, but the alcohol treatment of the grandparents does not seem responsible for any such hypothetical difference, since control rats as well as tests have 'failures'; the numbers are four controls and six tests, which, in such small numbers, is a very close approximation to equality. A second method of handling the data of the rats that had 'failures' would be to omit the time spent on 'failure' trials, assuming that the 'failure' was due to circumstances quite apart from the nature of the rat. But, as stated, this does not eliminate the effect of the 'failure' upon the immediately following trials.

Perhaps the best way to include the 'failure' rats is to put down a uniform time (ten minutes) for each 'failure' and treat that as a successful trial; then, in averaging, count the number of trials actually attempted. When a 'failure' occurred on the first or second trials, there were no more trials attempted that day. This method has been used for including the 'failure' rats in the two main series of summaries of the time data, although it is obviously not free from very real objection. In applying this to the summaries based on the individual days, a further adjustment was needed. Since the sum of the three trials for each rat was used instead of the average per trial, it was necessary to compensate in some way for the missing third trial when a 'failure' occurred on the second trial. This was done by increasing the sum of the first trial and the 'failure' (ten minutes) by one half of this sum. The two rats that lost a whole day through failures on the first trials have not been used in these averages.

Since no satisfactory method of including the data from these 'failure' rats has been discovered, the best method of meeting the situation seems to be to omit these data. Accordingly, for most of the criteria only the averages excluding these data are given. But, in order to show that the results are not dependent upon their exclusion, the averages for time, which may be considered the most important single criterion, are given both including and excluding these 'failure' rats. Theoretically, both sets



of averages might be demanded throughout, but the doubling of every table would result in unjustifiable bulk, especially in the light of the clearness of the results as they stand.

When it became necessary to make drastic reductions in the size of the experiment, eleven rats in this generation, six tests and five controls, were taken out of the training after they had been given the eight days of training. They lack the training in the multiple-choice apparatus as well as retention on the maze. In order to have the same group of rats summarized for the maze and the multiple-choice apparatus, these eleven rats were not included in the major part of the calculating that has been done with the maze data. However, from the standpoint of this paper, these rats, as far as they went, are as good as others; accordingly, they have been included in the most important summaries. The time data are summarized, including, as well as excluding, these rats which we call 'incompletes,' since their training was not completed. There are then three sets of rats to be designated 'completes,' 'incompletes,' and 'failures.'

#### *f. Irregularities of data*

The number of rats included in the various averages is indicated in the tables; these numbers vary for different averages even within one group of rats. The details explaining each of these irregularities are given in the following paragraphs. Although we feel fully justified in making the minor exclusions and inclusions besides the main ones treated above, we wish to remove all suspicion of our having tried to influence the result one way or the other in so doing. These explanations are made here all together to avoid many scattered foot-notes.

Rat 1279: Record sheets for the first day lost; this rat is not included in the averages for the first day or for *first half of training, all training, training and retention*.

Rat 1311: Record sheets for third day lost; this rat is not included in averages for the third day, *first half of training, all training, training and retention*.

Rat 1265: Time record for twenty-first trial lost; averages for *second half of training*, *all training*, *training and retention* exclude this trial. In obtaining total time for the seventh day of *training*, there was substituted for the twenty-first trial a time based on its distance record.

Rat 1211: Showed very unusual behavior on the second day of retention, taking five minutes when it might have been expected to take five seconds. The rat was obviously sick, so its records have been omitted from the average for *retention*. For *training and retention* its exclusion makes such a slight difference that only the averages including it have been given.

Rat 1312: Failed to reach the center on the first trial on the second day and did not succeed until the first trial on the fourth day.

Rat 1169: Failed on the first trial on the second day and succeeded on the first trial on the third day. These last two rats are the only cases where a whole day was lost through failure; to give the arbitrary maximum in these cases seemed to make the result depend too heavily upon the arbitrary figures. In one case only half of the average would be based on actual records and in the other, three-quarters would be based on actual records. These two rats are so outstandingly different from the other 'failure' rats that it has seemed more fair to omit them from the averages including the first three days. They are included in the averages for the *second half of training* and for *retention* when the other 'failure' rats are included.

Rats 1193, 1168, and 1173 had no perfect trials in all of the thirty-six trials of *training and retention*. In the summaries on the number of trials before the first perfect trial, these rats have been given a score of 36, as though they had had a perfect trial on the thirty-seventh trial (which was not given). To give averages free from this difficulty, these three rats are omitted from the alternative averages.

*g. Criteria employed*

The evidence about to be presented is derived from three sources: the time for each trial, the distance covered on each trial, the number of departures from the true path, or errors. The time and distance data make it possible to calculate the speed or the distance covered per second; from the error data the occurrence of perfect or errorless trials is obtained. The number of perfect trials and the number of days before the first perfect trial can be used as criteria, since all the rats received the same number of trials. Under these main headings the test and control rats have been compared in various groupings of the strains and sexes, for various periods of the training.

The question of the differences between the behavior of the males and females presents a difficulty. Although we have made extended studies of this question on the animals so far summarized, we offer no conclusion. Since we have a much larger mass of data still awaiting analysis, namely, the records of the rats that were actually treated with alcohol and their immediate offspring, we withhold judgment for the present and give summaries with the males and females separately and together.

## RESULTS

*1. Morphological characters*

Although this paper is on behavior, it may be of interest to note here that we have found, as the result of alcoholizing the rats, no signs of such morphological abnormalities as Stockard and Papanicolaou ('16 and '18) found in the descendants of alcoholized guinea-pigs. The few abnormalities that have appeared have been limited to one strain; they have appeared both in control and test rats. Very obviously, these abnormalities were carried in the strain and were not induced by alcohol. The immediate effect of the alcohol upon the treated animals was to render the hair exceptionally fine and attractive by keeping off all skin parasites.

## 2. Comparison of the test and control rats on the basis of time

a. *Time: averages per different groups of trials.* The time data give the number of seconds taken in passing from the entrance of the maze to the doorway of the central food compartment. The observer sat with eyes on the record sheet within the dark box and the left hand on the stop-watch, which was fixed in position on the table by its holder; when the rat was observed to enter the maze the watch was started and it was stopped when the rat entered the final door. Of the various criteria, time is probably most free from errors in observation; the beginning and end of a trial were clearly marked events that could be measured accurately within the limits of the time of the observer's response to a sensory image.

The first table is based on the average time per trial of each rat for the following groups of trials: *first half of training* (12 trials), *second half of training* (12 trials), *omitting the first day* (21 trials), *all training* (24 trials), *retention* (12 trials), and *training and retention* (36 trials). The first division of the table gives the averages for the group of rats that were given full training, 'completes'; in the second division the averages include the rats that were not given full training, 'incompletes' as well as the 'completes'; the third division of the table includes the 'failures,' the 'completes' and the 'incompletes.' Each number in the body of the table is an average per trial in seconds of the averages for each rat; the number of rats involved in each average is given. Since there is a great difference in the time taken in different parts of the training, the tests and controls are compared by means of ratios instead of by differences. In every case the larger of the pair of averages has been divided by the smaller number, thus always giving ratios above 1.000. When the test average is larger, that is, the tests taking more time, the ratio is called 'plus'; and when the control average is larger, that is, the controls taking more time than the tests, the ratio is called 'minus.' Accordingly, a ratio of (+)1.500 indicates that the test average is one and a half times as large as the comparable control average; the tests took half as long again



TABLE 1

*Time per trial in seconds for six groupings of the trials; average of the averages for each rat, calculated from table A in the appendix. 'Completes' were given complete training; 'incompletes' did not have the complete training, and 'failures' had an unfinished trial. For each pair of test and control averages a ratio is given: a 'plus' ratio indicates that the test average is higher and has been divided by the control average; while a 'minus' ratio indicates that the control average is higher and has been divided by the test average. The number of rats involved is shown above each set of averages*

STRAIN	GROUP OF TRIALS	'COMPLETES'									
		Males			Females			Males and females			
		Tests	Controls	Ratio	Tests	Controls	Ratio	Tests	Controls	Ratio	
A	First half training .....	3 rats	3 rats	(+)1.590	3 rats	4 rats	(+)1.077	6 rats	7 rats	(+)1.408	
	Second half training .....	184.83	116.26	(+)1.843	117.39 <sup>1</sup>	108.92	(+)1.157	157.86 <sup>1</sup>	112.06	(+)1.359	
	Omitting first day .....	33.77	18.32	(+)1.646	36.24	31.31	(+)1.206	35.01	25.74	(+)1.383	
	All training .....	47.91	29.11	(+)1.607	43.21	35.81	(+)1.113	45.56	32.94	(+)1.399	
	Retention .....	109.30	67.98	(-)1.100	78.10 <sup>1</sup>	70.11	(+)1.064	96.82 <sup>1</sup>	69.20	(-)1.011	
C	Training and retention .....	24.05	26.47	(+)1.494	25.66	24.12	(+)1.088	24.85	25.13	(+)1.327	
	First half training .....	80.88	54.14	(+)1.246	59.63 <sup>1</sup>	54.78	(+)1.074	72.38 <sup>1</sup>	54.51	(+)1.080	
	Second half training .....	3 rats	3 rats	(+)1.666	1 rat	2 rats	(+)2.579	4 rats	5 rats	(+)1.918	
	Omitting first day*	127.98	102.70	(+)1.359	193.06	179.75	(-)1.009	144.25	133.52	(+)1.099	
	All training .....	18.27	10.96	(+)1.286	22.93	8.89	(+)1.256	19.43	10.13	(-)1.066	
L	Retention .....	26.72	19.66	(-)1.192	37.09	37.42	(+)1.535	29.31	26.65	(+)1.055	
	Training and retention .....	73.12	56.83	(+)1.094	108.00	94.32	(+)1.876	81.84	71.83	(+)1.139	
	First half training .....	12.51	14.91 <sup>2</sup>	(+)1.660	19.53	15.54	(+)1.390	14.27	15.22 <sup>2</sup>	(-)1.066	
	Second half training .....	52.92	48.38	(+)1.047	78.51	68.06	(+)1.728	59.31	56.21	(+)1.055	
	Omitting first day .....	4 rats	2 rats	(+)1.590	5 rats	6 rats	(+)1.805	9 rats	8 rats	(+)1.797	
	Retention .....	282.33 <sup>3</sup>	170.09	(+)1.638	213.37	113.68	(+)1.623	239.23 <sup>3</sup>	127.77	(+)1.872	
	Training and retention .....	29.05	27.73	(+)1.601	26.96	19.39	(+)1.851	27.89	21.48	(+)1.298	
	First half training .....	46.02 <sup>3</sup>	29.45	(+)1.590	48.54	28.08	(+)1.876	47.60 <sup>3</sup>	28.42	(+)1.674	
	Second half training .....	157.31 <sup>3</sup>	98.92	(+)1.638	120.16	66.54	(+)1.805	134.11 <sup>3</sup>	74.63	(+)1.797	
	Omitting first day .....	25.73	15.70	(+)1.601	23.18	14.28	(+)1.623	24.31	14.64	(+)1.660	
	Retention .....	114.21 <sup>3</sup>	71.18	(+)1.601	87.83	47.45	(+)1.851	97.73 <sup>3</sup>	54.63	(+)1.789	
	Training and retention .....										

<sup>1</sup> Omitting rat 1279: record of first day lost. <sup>2</sup> Omitting rat 1211 (sick). <sup>3</sup> Omitting rat 1311: record of third day lost.

	COMPLETES <sup>1</sup> AND 'INCOMPLETES'					
	10 rats	8 rats	9 rats	12 rats	19 rats	20 rats
All {						
First half training.....	198.38	124.63	(+)-1.592	186.83 <sup>1</sup>	(+)-1.517	192.95 <sup>1,3</sup>
Second half training.....	27.23	17.91	(+)-1.520	29.61	(+)-1.370	20.13
Omitting first day.....	40.22	25.65	(+)-1.568	45.49	(+)-1.412	29.90
All training.....	113.24	71.53	(+)-1.583	108.13	(+)-1.491	110.83 <sup>3,1</sup>
Retention.....	21.26	20.09 <sup>2</sup>	(+)-1.058	23.61	(+)-1.328	18.63 <sup>2</sup>
Training and retention....	82.67	56.24	(+)-1.469	79.62	(+)-1.470	54.66
A {						
First half training.....	217.83	116.26	(+)-1.873	117.39 <sup>1</sup>	(-)-1.022	118.91
Second half training.....	32.20	18.32	(+)-1.757	36.24	(-)-1.118	33.86
Omitting first day.....	57.49	29.11	(+)-1.974	43.21	(+)-1.093	36.40
All training.....	125.01	67.98	(+)-1.839	78.10 <sup>1</sup>	(-)-1.028	76.59
Retention.....						
Training and retention....						
C {						
First half training.....	4 rats	3 rats	1 rat	2 rats	5 rats	5 rats
Second half training.....	144.10	102.70	(+)-1.403	193.06	(+)-1.074	133.52
Omitting first day.....	15.96	10.96	(+)-1.456	22.93	(+)-2.579	10.13
All training.....	24.04	19.66	(+)-1.223	37.09	(-)-1.009	26.65
Retention.....	80.03	56.83	(+)-1.408	108.00	(+)-1.256	71.83
Training and retention....						
L {						
First half training.....	7 rats	4 rats	5 rats	6 rats	12 rats	10 rats
Second half training.....	269.91 <sup>3</sup>	171.06	(+)-1.578	213.37	(+)-1.877	136.63
Omitting first day.....	35.33	25.91	(+)-1.363	26.96	(+)-1.390	22.00
All training.....	53.22 <sup>3</sup>	30.75	(+)-1.731	48.54	(+)-1.728	29.14
Retention.....	153.95 <sup>3</sup>	98.49	(+)-1.563	120.16	(+)-1.805	79.32
Training and retention....						
All {						
First half training.....	16 rats	10 rats	9 rats	15 rats	25 rats	25 rats
Second half training.....	219.00 <sup>3</sup>	134.11	(+)-1.633	186.83 <sup>1</sup>	(+)-1.489	128.92
Omitting first day.....	29.51	19.15	(+)-1.541	27.55	(-)-1.010	24.37
All training.....	46.86 <sup>3</sup>	26.93	(+)-1.740	45.49	(+)-1.312	31.57
Retention.....	124.59 <sup>3</sup>	76.84	(+)-1.621	108.13 <sup>1</sup>	(+)-1.410	76.73
Training and retention....						

TABLE 1—Continued

STRAIN	GROUP OF TRIALS	'COMPLETES,' 'INCOMPLETES,' AND 'FAILURES'								
		Males			Females			Males and females		
		Tests	Controls	Ratio	Tests	Controls	Ratio	Tests	Controls	Ratio
A	First half training.....	6 rats 217.83	3 rats 116.26	(+) 1.873	5 rats 201.90 <sup>1</sup>	7 rats 120.04	(+) 1.681	11 rats 210.74 <sup>1</sup>	10 rats 118.91	(+) 1.772
	Second half training.....	31.60	18.32	(+) 1.725	33.53	40.52	(-) 1.208	32.48	33.86	(-) 1.042
	Omitting first day.....	57.49	29.11	(+) 1.975	64.31	39.52	(+) 1.627	60.90	36.40	(+) 1.673
	All training.....	125.01	67.98	(+) 1.839	115.23 <sup>1</sup>	80.28	(+) 1.435	120.66 <sup>1</sup>	76.59	(+) 1.575
	Retention.....	21.09	26.47	(-) 1.255	25.66	24.12	(+) 1.063	23.05	25.13	(-) 1.090
C	Training and retention...	80.88	54.14	(+) 1.494	59.63 <sup>1</sup>	54.78	(+) 1.088	72.38 <sup>1</sup>	54.51	(+) 1.327
	First half training.....	4 rats 144.10	3 rats 102.70	(+) 1.403	2 rats 183.34	2 rats 179.75	(+) 1.020	6 rats 157.18	5 rats 133.52	(+) 1.177
	Second half training.....	15.96	10.96	(+) 1.456	19.80	8.89	(+) 2.227	17.24	10.13	(+) 1.702
	Omitting first day.....	24.04	19.66	(+) 1.223	37.06	37.42	(-) 1.010	28.38	26.65	(+) 1.065
	All training.....	80.03	56.83	(+) 1.408	101.57	94.32	(+) 1.077	87.21	71.83	(+) 1.214
L	Retention.....	12.51	14.91 <sup>2</sup>	(-) 1.192	16.59	15.54	(+) 1.067	14.15	15.22 <sup>2</sup>	(-) 1.076
	Training and retention...	52.92	48.38	(+) 1.094	73.24	68.06	(+) 1.076	61.05	56.21	(+) 1.086
	First half training.....	7 rats 269.91 <sup>3</sup>	7 rats 178.84	(+) 1.509	7 rats 212.04	7 rats 164.19	(+) 1.291	14 rats 240.97 <sup>3</sup>	14 rats 171.52	(+) 1.405
	Second half training.....	35.33	27.56	(+) 1.282	25.49	18.82	(+) 1.354	30.41	23.19	(+) 1.311
	Omitting first day.....	53.22 <sup>3</sup>	40.08	(+) 1.328	50.51	42.89	(+) 1.178	51.87 <sup>3</sup>	41.49	(+) 1.250
All	All training.....	153.95 <sup>3</sup>	102.26	(+) 1.505	119.40	91.51	(+) 1.305	136.68 <sup>3</sup>	96.89	(+) 1.410
	Retention.....	25.73	15.12	(+) 1.701	24.99	17.30	(+) 1.444	25.26	16.52	(+) 1.529
	Training and retention...	114.21 <sup>3</sup>	71.22	(+) 1.604	87.94	66.77	(+) 1.317	96.70 <sup>3</sup>	68.39	(+) 1.414
	First half training.....	17 rats 219.00 <sup>3</sup>	13 rats 146.83	(+) 1.491	14 rats 203.88 <sup>1</sup>	16 rats 146.82	(+) 1.388	31 rats 212.28 <sup>1,3</sup>	29 rats 146.83	(+) 1.445
	Second half training.....	29.46	21.60	(+) 1.364	27.55	27.07	(+) 1.018	28.60	24.62	(+) 1.161
All	Omitting first day.....	46.86 <sup>3</sup>	32.84	(+) 1.427	53.75	40.73	(+) 1.319	50.06 <sup>3</sup>	37.19	(+) 1.346
	All training.....	124.59 <sup>3</sup>	83.87	(+) 1.485	115.04 <sup>1</sup>	86.95	(+) 1.323	120.35 <sup>1,3</sup>	85.57	(+) 1.406
	Retention.....	21.35	18.86 <sup>2</sup>	(+) 1.132	23.76	19.13	(+) 1.242	22.61	19.02 <sup>2</sup>	(+) 1.188
	Training and retention...	82.67 <sup>3</sup>	59.25	(+) 1.395	79.34 <sup>1</sup>	63.28	(+) 1.254	80.92 <sup>1,3</sup>	61.53	(+) 1.315

<sup>1</sup> Omitting rat 1279: record of first day lost. <sup>2</sup> Omitting rat 1211 (sick). <sup>3</sup> Omitting rat 1311: record of third day lost.

Note: In group 'including failures' there are, for retention and training and retention, 23 tests and 23 controls.

as the controls. A ratio of  $(-)$ 1.500 indicates that the controls took half as long again as the tests.

The males give 'plus' ratios for each group of the trials in *training*, whether the strains are given separately or together. In *retention* one strain gives 'plus' ratios and two strains give 'minus' ratios, but when all strains are averaged together and rat 1211 omitted (page 219), the ratio becomes 'plus'  $((+)$ 1.058 for 'completes' and  $(+)$ 1.132 when the 'failures' are added).

The females give fifty-six 'plus' ratios out of sixty-four when all the different combinations of the data are counted. In the group of 'completes,' strain C gives a ratio very slightly 'minus'  $((-)$ 1.009) for *omitting the first day*, but it will be noted that there is only one test rat and two controls involved in this ratio. When all the strains are combined, this period (*omitting the first day*) gives 'plus' ratios of  $(+)$ 1.412,  $(+)$ 1.312, and  $(+)$ 1.319 for each of the three sets of rats, respectively. In strain A the 'completes' and 'incompletes' give slightly 'minus' ratios for the first and second halves of training, and so for *all training* as well. When the 'failure' rats are also included, the 'minus' ratio in strain A for the *second half of training* remains, but the ratios for the *first half of training* and for *all training* become 'plus.' All strains averaged together give 'plus' ratios for each group of trials and each set of rats, excepting the second half of training for the 'completes' and 'incompletes.' When the 'failure' rats are also added, the ratio for the *second half of training* becomes 'plus'  $((+)$ 1.018); all the other ratios are over  $(+)$ 1.200. Combining all the data for the females by averaging the averages of each rat's thirty-six trials in *training and retention*, all strains together, the group of 'completes' gives a ratio of  $(+)$ 1.470; 'completes' and 'failures' give  $(+)$ 1.254.

The test males took more time than the controls in every case in *training*, but in *retention* the control males in two strains took more time than the tests; the females, on the other hand, show the tests taking more time in every case in *retention*, while in *training* they show a few cases where the controls took slightly more time. When the strains are averaged together, all the groups of trials show the test averages clearly greater than the



control averages, excepting *retention* for the males, and the *second half of training* for the females; these two pairs of averages give ratios very close to 1.000.

Combining the sexes (primary averaging, disregarding sex) and taking each strain separately, all the ratios are 'plus' excepting *retention* for strains A and C, and the *second half of training* for strain A when the 'incompletes' are included with and without the 'failures.' The highest of these 'minus' ratios is (-)1.090.

When the strains as well as the sexes are averaged together, 'plus' ratios are given in every case. The differences and the probable errors of the differences between the averages are given in table 2. (The probable error of the difference is the square root of the sum of the squares of the probable errors of the two averages compared.) In a separate column are given the quotients of the differences divided by their probable errors. A quotient of three or more is usually regarded by statisticians as certainly indicating a significant difference. In the *first half of training*, *omitting the first day*, *all training*, and *training and retention* the differences are more than three times their probable errors and may be considered to be real or significant differences. In the *second half of training* the 'completes' alone give a significant difference, when the 'incompletes' and when the 'failures' are included, the differences are 1.99 and 1.74 times their probable errors; in *retention* the differences are 2.06 and 2.24 times their probable errors. So besides finding many more 'plus' than 'minus' ratios, the differences are shown to be statistically significant when grouped to obtain large enough numbers to calculate probable errors.

The frequency distribution of the averages for each rat, including 'failures,' from which the averages in the preceding tables were obtained, are shown graphically in figure 2. For each group of trials there is a pair of overlapping curves; the solid line represents the controls, the broken line the tests. The number of rats in the test and control groups is so nearly equal that a reduction to a percentage basis is not necessary. The graphs show a clear tendency for the distribution of the tests to lie further up the scale than the distributions of the controls; at

TABLE 2

*Showing the significance of the differences between the averages of the time taken by the tests and controls for each of the six groupings of the trials; sexes together and strains together; with the probable errors of the differences and the differences in terms of (divided by) their probable errors. A plus difference indicates that the test average is the larger (longer time). When the difference is three or more times its probable error it may be considered significant*

GROUP OF TRIALS	'COMPLETES'			'COMPLETES' AND 'INCOMPLETES'			'COMPLETES,' 'INCOMPLETES' AND 'FAILURES'		
	Diff.	P. E.	Diff./P. E.	Diff.	P. E.	Diff./P. E.	Diff.	P. E.	Diff./P. E.
First half training . . . . .	+69.24±16.94		4.10	+78.89±5.87		4.97	+65.45±14.59		4.48
Second half training . . . . .	+8.23±2.34		3.52	+5.18±2.60		1.99	+3.98±2.38		1.74
Omitting first day . . . . .	+12.77±3.17		4.03	+14.78±3.20		4.61	+12.87±4.04		3.18
All training . . . . .	+38.80±8.38		4.63	+42.14±7.21		5.84	+34.78±7.46		4.66
Retention . . . . .	+3.74±1.81		2.06				+3.59±1.60		2.24
Training and retention . . . . .	+26.57±6.00		4.43				+19.39±6.22		3.12

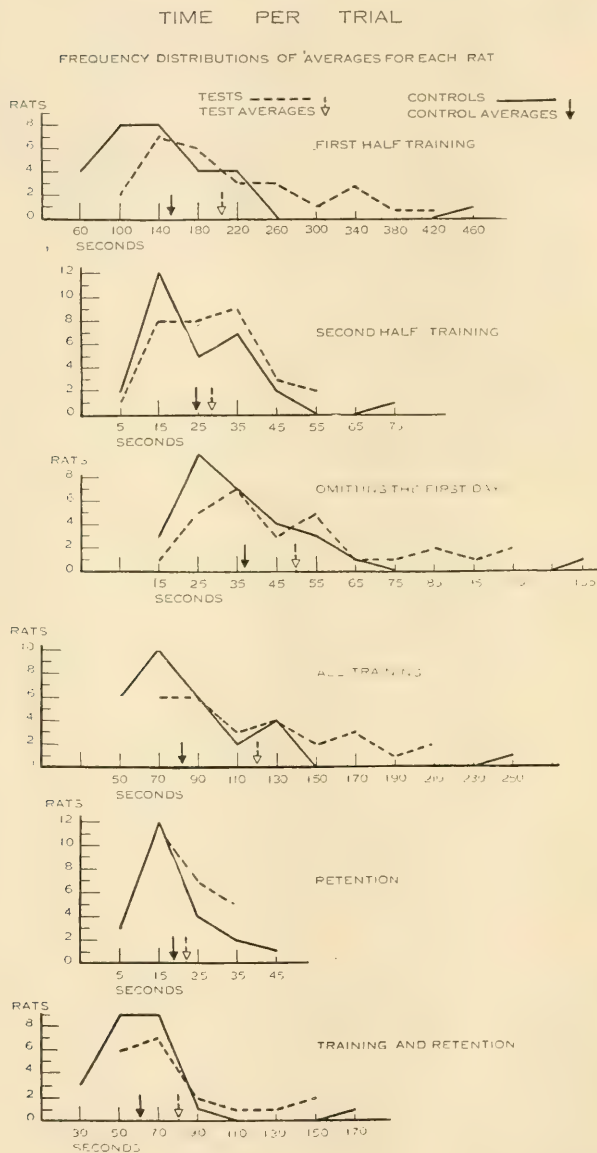


Fig. 2 Frequency distributions of the averages for each rat for time per trial, based on six different groupings of the trials. The broken lines are the tests; the solid lines, the controls. Units on the base line are seconds, units on the vertical scale are numbers of rats. 'Completes,' 'incompletes,' and 'failures' are used with the males and females in all strains.

the lower end the controls are more numerous, at the upper end, the tests. This is evidence that the means are good indices of these two distributions; that the differences between the two groups of rats, tests and controls, as given by their averages, are real and not due to some peculiarity of the frequency distributions.

There can be no question but that there was a tendency for the test rats to spend more time in learning the maze than did the controls. That the differences in their averages is not significant in *retention* and in the *second half of training* when the 'incompletes' and 'failures' are included, in no way weakens this conclusion. The maze was simple enough to be learned by all rats, and the number of trials given was great enough to include overtraining for many of the rats; that is, many rats were continued after they had stopped improving their average time. Longer training would probably have removed the difference between the averages of the tests and controls. In the number of trials given, the differences between the averages was enough reduced in the last part of the period of training to fall within the range of chance variation. The averages for each rat from which the preceding summaries have been made are given in table A in the appendix.

*b. Time: averages for each day.* The next step in the analysis of the time data is the comparison of the averages of the tests and controls on each of the eight days of *training* and the four days of *retention*. This series of calculations is based on the total time spent each day by each rat in running its three trials. Instead of using averages based on groups of 12, 24, or 36 trials, the sums of the three trials on each day are now used. The succession of the averages of these sums gives a curve (learning curve) that shows the daily reduction in the time required to make three successive trips through the maze. Such a curve for all the strains and both sexes together is given in figure 3. This curve shows that the tests (broken line) are above the controls (solid line), that is, took more time on each day of *training* and on all but the first day in *retention*. The light lines show the tests and controls when the 'failure' rats are included. The



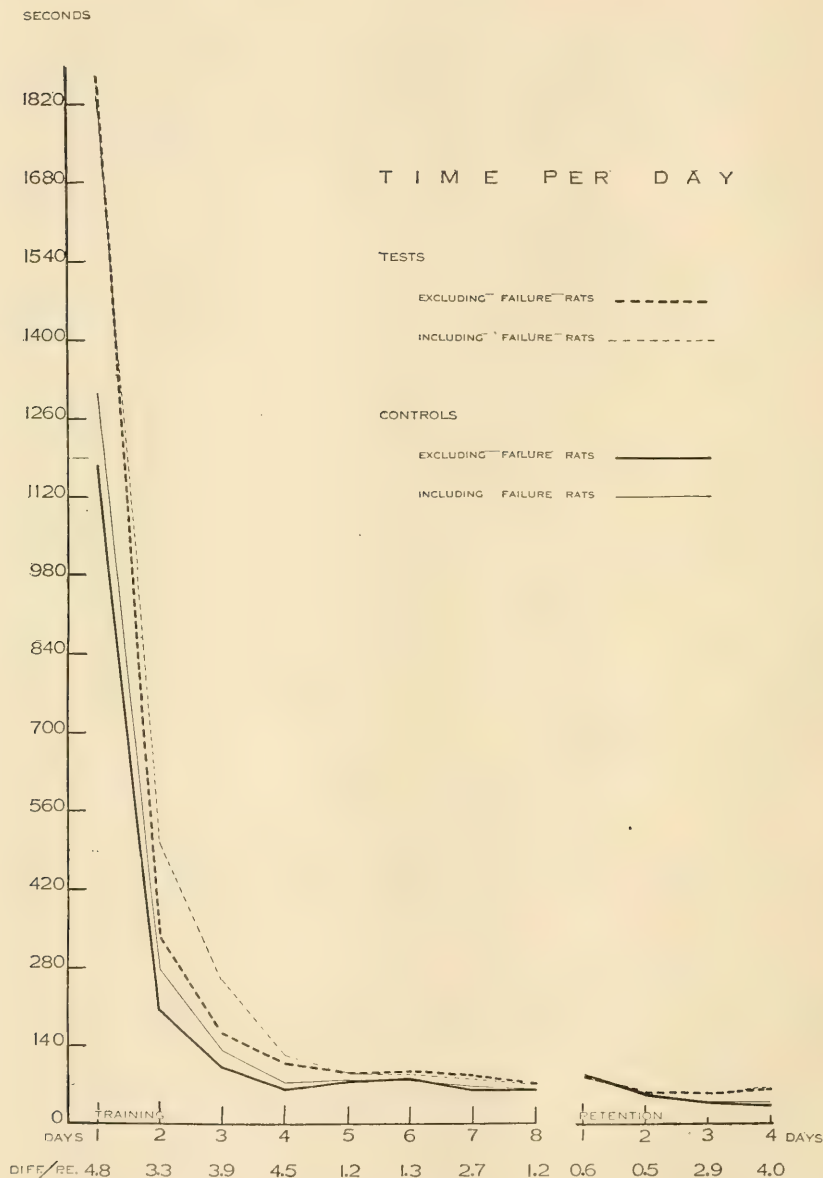


Fig. 3 Learning curve based on time; averages per trial on each day of *training* and *retention*. The broken lines are tests; the solid lines, controls; the heavy lines based on 'completes' and 'incompletes,' the light lines include 'failures' as well. Males and females in all strains are put together in all cases. The units on the base lines are days: the first series of days are *training*; the second series, *retention*; units on the vertical scale are seconds.

number below each day is the number of times the difference between the test and control averages is greater than its probable error, considering just the 'completes' and 'incompletes.' The data for this figure are given in table 3.

The first half of table 3 is based on the 'completes' and 'incompletes,' the second half, at the right, includes the 'failures' as well. 'Completes' averaged alone are not given; they show substantially the same results as when averaged with 'incompletes.' In each half of the table there are three main sections: 1, males; 2, females; 3, males and females (i.e., primary data put together regardless of sex, not averages of the averages of each sex). Under each of these headings the time per rat for the tests and controls is given for each day (eight days of training and then four days of retention). The three strains are considered separately, then all strains together. The number of rats in each series of averages is shown at the top of the series. As before, the averages are compared by ratios; plus ratios indicate that the tests took longer time than the controls; minus ratios indicate that the controls took longer time than the tests.

If just the signs of the ratios are studied, it is obvious that there is a great preponderance of 'plus' signs. When the 'completes' and 'incompletes' are considered, the males give 44 'plus' ratios out of 48; the females, 38 'plus' ratios out of 48; and the males and females together, 40 'plus' ratios out of 48. When the 'failures' are included, out of 48 cases the males give 42 'plus' ratios, the females 37 'plus' ratios, and the males and females together 40 'plus' ratios. In the whole table there are 241 'plus' ratios out of a possible 288. This is, of course, only a general comparison, since in many of these averages the same data are repeated, but it indicates that combining the data in different ways gives the same result. In other words, the greater time taken by the tests is a real difference and not due to the special method of treating the data. Some of the ratios of tests vs. controls given in table 3 are shown graphically in figure 4; the sexes together, each strain separately, and all strains together. The straight horizontal line represents equality between the test and control average, or the ratio of 1.000.

TABLE 3

Time in seconds for each day of the training; averages of the total time spent on each day by each rat. The averages are compared by ratios; a plus ratio indicates that the test average is higher and has been divided by the control average, while a minus ratio indicates that the control average is higher and has been divided by the test average. The great preponderance of plus ratios shows that there is a strong tendency for the tests to take more time than the controls day by day

DAY		'COMPLETES' AND 'INCOMPLETES'								
		Males			Females			Males and females		
		Tests	Controls	Ratios	Tests	Controls	Ratios	Tests	Controls	Ratios
Strain A		5 rats	3 rats		3 rats	7 rats		8 rats	10 rats	
	1	1793.0	1005.7	(+ )1.789	867.3 <sup>1</sup>	1096.8	(- )1.264	1528.5 <sup>1</sup>	1069.3	(+ )1.429
	2	540.6	191.3	(+ )2.825	195.5	165.4	(+ )1.181	411.1	173.2	(+ )2.373
	3	161.5	139.6	(+ )1.156	183.8	98.3	(+ )1.869	169.9	110.7	(+ )1.534
	4	118.8	59.1	(+ )2.010	93.2	79.9	(+ )1.166	109.2	73.7	(+ )1.481
	5	138.9	54.5	(+ )2.548	113.8	141.1	(- )1.239	129.5	115.1	(+ )1.125
	6	105.3	52.0	(+ )2.025	98.2	140.1	(- )1.426	102.6	113.7	(- )1.108
	7	71.7	51.2	(+ )1.400	120.1	114.9	(+ )1.045	89.8	95.8	(- )1.066
Strain B	8	70.5	55.9	(+ )1.261	102.9	90.0	(+ )1.143	82.6	79.8	(+ )1.035
		3 rats	3 rats		3 rats	4 rats		6 rats	7 rats	
	1	72.2	117.4	(- )1.626	98.4	107.0	(- )1.087	85.4	111.5	(- )1.305
	2	69.3	104.6	(- )1.509	77.6	55.2	(+ )1.405	73.4	76.4	(- )1.040
	3	65.4	55.5	(+ )1.178	55.7	65.2	(- )1.170	60.5	61.1	(- )1.009
	4	81.6	40.0	(+ )2.040	76.1	62.0	(+ )1.227	78.8	52.6	(+ )1.498
		4 rats	3 rats		1 rat	2 rats		5 rats	5 rats	
	1	1415.8	951.0	(+ )1.488	1813.0	1477.8	(+ )1.226	1495.2	1161.7	(+ )1.287
Strain C	2	158.7	150.5	(+ )1.054	308.6	567.6	(- )1.839	188.6	317.3	(- )1.682
	3	94.7	86.9	(+ )1.089	123.6	71.0	(+ )1.740	100.5	80.5	(+ )1.248
	4	59.8	44.1	(+ )1.356	71.6	40.7	(+ )1.759	62.2	42.7	(+ )1.456
	5	65.4	43.5	(+ )1.503	148.6	28.1	(+ )5.288	82.1	37.3	(+ )2.201
	6	49.3	28.1	(+ )1.133	58.4	26.5	(+ )2.203	51.1	27.5	(+ )1.858
	7	40.5	29.0	(+ )1.396	45.4	26.1	(+ )1.739	41.5	27.8	(+ )1.492
	8	36.2	30.9	(+ )1.171	22.8	26.0	(- )1.140	33.5	28.9	(+ )1.159

<sup>1</sup> Omitting rat 1270; record for eighth day lost.

<sup>2</sup> Omitting rat 1311; record for third day lost.

<sup>3</sup> Omitting rat 1211; sick.

		3 rats	3 rats	1 rat	2 rats	4 rats	5 rats	
Retention.....		1	45.5	(- )2.338	36.4	(- )1.741	43.2	
		2	28.2	(+ )1.025	109.0	58.6	(+ )1.860	
		3	46.5	(+ )1.422	57.6	21.8	(+ )2.642	
		4	30.1	(+ )1.400	31.4	42.7	(- )1.359	
Strain L								
Training.....		7 rats	4 rats	5 rats	6 rats	12 rats	10 rats	
		1	2457.7	(+ )1.430	1864.5	1007.3	(+ )1.850	2210.5
		2	330.4	(+ )2.699	361.7	212.8	(+ )1.699	343.4
		3	192.5 <sup>2</sup>	(+ )1.483	182.5	84.8	(+ )2.152	172.3
		4	115.8	(+ )1.398	151.7	59.3	(+ )2.558	130.8
		5	83.0	(+ )1.034	59.8	52.3	(+ )1.143	73.3
		6	137.5	(+ )1.375	81.6	69.1	(+ )1.180	114.2
		7	109.9	(+ )1.623	112.2	46.1	(+ )2.433	110.9
Retention.....		8	93.5	(+ )1.491	69.8	65.1	(+ )1.072	
		9 rats	3 rats	5 rats	6 rats	9 rats	8 rats	
		1	126.0	(+ )1.262	94.3	70.9	(+ )1.330	108.4
		2	66.3	(+ )1.560	43.6	42.2	(+ )1.033	53.7
All strains		3	74.1	(+ )2.704	44.9	24.8	(+ )1.803	
		4	42.4	(+ )2.207	95.4	33.3	(+ )2.804	57.8
		16 rats	10 rats	9 rats	15 rats	25 rats	25 rats	
		1	1989.5	(+ )1.561	1608.8 <sup>1</sup>	1111.8	(+ )1.447	1862.6 <sup>1</sup>
Training.....		2	353.2	(+ )2.331	300.4	238.0	(+ )1.262	
		3	156.1 <sup>2</sup>	(+ )1.301	176.4	89.3	(+ )1.975	334.2
		4	102.7	(+ )1.604	123.3	66.4	(+ )1.856	163.7 <sup>2</sup>
		5	96.1	(+ )1.562	87.7	90.5	(+ )1.031	110.2
Retention.....		6	105.4	(+ )1.646	84.6	96.6	(- )1.141	
		7	80.6	(+ )1.577	107.4	75.5	(+ )1.422	97.9
		8	71.9	(+ )1.407	75.6	71.5	(+ )1.057	90.3
		19 rats	8 rats	9 rats	12 rats	19 rats	20 rats	
Training.....		1	85.7	(+ )1.270	89.3	81.7	(+ )1.093	
		2	55.8	(- )1.163	62.3	49.3	(+ )1.203	87.4
		3	63.2	(+ )1.583	49.9	37.8	(+ )1.320	58.8
		4	50.5	(+ )1.823	81.9	44.5	(+ )1.840	56.9
Retention.....		5	27.7	(+ )1.840	65.3	37.8	(+ )1.474	
		6	37.8	(+ )1.474	37.8	37.8	(+ )1.474	37.8
		7	37.8	(+ )1.474	37.8	37.8	(+ )1.474	37.8
		8	37.8	(+ )1.474	37.8	37.8	(+ )1.474	37.8



TABLE 3—Continued  
'COMPLETES,' 'INCOMPLETES,' AND 'FAILURES'

DAY		Males			Females			Males and females		
		Males			Females			Males and females		
		Tests	Controls	Ratios	Tests	Controls	Ratios	Tests	Controls	Ratios
Strain A		6 rats 1677.9	3 rats 1005.7	(+1.088	5 rats 1550.4 <sup>1</sup>	7 rats 1096.8	(+1.413	11 rats 1626.9 <sup>1</sup>	10 rats 1069.3	(+1.521
1		750.5	191.3	(+3.923	648.3	165.4	(+3.919	704.0	173.2	(+4.064
2		266.1	139.6	(+1.906	198.6	98.3	(+2.020	235.4	110.7	(+2.126
3	Training.....	127.9	59.1	(+2.164	101.2	79.9	(+1.266	115.7	73.7	(+1.569
4		140.4	74.5	(+2.576	119.9	141.1	(-1.176	131.1	115.1	(+1.139
5		97.0	52.0	(+1.865	90.8	140.1	(-1.542	91.2	113.7	(+1.207
6		70.8	51.2	(+1.382	105.3	114.9	(-1.091	86.4	95.8	(-1.108
7		71.1	55.9	(+1.271	86.4	90.0	(-1.041	78.0	79.8	(-1.023
8										
Strain C		4 rats 71.1	3 rats 117.4	(-1.651	3 rats 98.4	4 rats 107.0	(-1.087	7 rats 82.8	7 rats 111.5	(-1.334
1	Retention.....	58.7	104.6	(-1.781	77.6	55.2	(+1.405	67.1	76.4	(-1.138
2		56.2	55.5	(+1.012	55.7	65.2	(-1.170	56.0	61.1	(-1.091
3		67.1	40.0	(+1.677	76.1	62.0	(+1.227	70.9	52.6	(+1.347
4										
Strain C		4 rats 1415.8	3 rats 951.0	(+1.488	2 rats 1659.5	2 rats 1477.8	(+1.122	6 rats 1497.0	5 rats 1161.7	(+1.288
1	Training.....	158.7	150.5	(+1.054	400.3	567.6	(-1.427	239.2	327.3	(-1.326
2		94.7	86.9	(+1.089	90.6	71.0	(+1.276	93.3	80.5	(+1.159
3		59.8	44.1	(+1.365	49.8	40.7	(+1.223	56.5	42.7	(+1.323
4		65.4	43.5	(+1.503	93.2	28.1	(+3.316	74.7	37.3	(+2.002
5		49.3	28.1	(+1.754	55.0	26.5	(+2.075	51.2	27.5	(+1.861
6		40.5	29.0	(+1.396	53.2	26.1	(+2.038	44.8	27.8	(+1.611
7		36.2	30.9	(+1.171	36.3	26.0	(+1.396	36.2	28.9	(+1.252
8										
Strain C		3 rats 45.5	3 rats 106.4	(-2.338	2 rats 35.1	2 rats 63.4	(-1.806	5 rats 41.3	5 rats 89.2	(-2.159
1	Retention.....	28.2	27.5 <sup>3</sup>	(+1.025	66.2	58.6	(+1.129	43.4	43.0 <sup>3</sup>	(+1.009
2		46.5	32.7	(+1.422	46.1	21.8	(+2.114	46.4	28.3	(+1.639
3		30.1	21.5	(+1.400	57.8	42.7	(+1.353	41.1	30.0	(+1.370
4										

Strain L		7 rats						7 rats		14 rats		14 rats	
		2457.7	1755.9	(+1.399	1805.4	1295.4	(+1.393	2131.5	1525.6	(+1.397	1525.6	(+1.397	(+1.397
Training.....	1	330.4	265.6	(+1.243	599.6	408.8	(+1.246	465.0	337.2	(+1.379	337.2	(+1.379	(+1.379
	2	192.5 <sup>2</sup>	134.4	(+1.432	429.8	202.3	(+2.124	320.3 <sup>2</sup>	168.3	(+1.903	168.3	(+1.903	(+1.903
	3	115.8	111.2	(+1.041	208.1	63.9	(+3.256	162.0	87.5	(+1.851	87.5	(+1.851	(+1.851
	4	83.0	84.5	(-1.018	62.4	52.4	(+1.190	72.7	68.5	(+1.061	68.5	(+1.061	(+1.061
	5	137.5	92.8	(+1.481	74.0	64.1	(+1.154	105.7	78.4	(+1.348	78.4	(+1.348	(+1.348
	6	109.9	78.5	(+1.400	93.0	48.0	(+1.937	101.5	63.3	(+1.603	63.3	(+1.603	(+1.603
	7	93.5	74.7	(+1.251	76.6	61.5	(+1.245	85.0	68.1	(+1.248	68.1	(+1.248	(+1.248
	8	4 rats	4 rats		7 rats	7 rats		11 rats	11 rats		11 rats		
Retention.....	1	126.0	81.7	(+1.542	107.6	88.2	(+1.219	114.3	85.8	(+1.332	85.8	(+1.332	(+1.332
	2	66.3	43.0	(+1.541	45.1	48.2	(-1.068	52.8	46.3	(+1.140	46.3	(+1.140	(+1.140
	3	74.1	28.1	(+2.637	41.6	30.9	(+1.346	53.4	29.9	(+1.785	29.9	(+1.785	(+1.785
	4	42.4	28.7	(+1.477	105.6	40.2	(+2.626	82.6	36.0	(+2.294	36.0	(+2.294	(+2.294
All strains		17 rats	13 rats		14 rats	16 rats		31 rats	29 rats		29 rats		
	1	1937.3	1396.9	(+1.386	1704.5	1231.3	(+1.384	1836.4 <sup>2</sup>	1305.5	(+1.406	1305.5	(+1.406	(+1.406
	2	438.3	221.9	(+1.975	588.5	322.2	(+1.826	506.1	277.2	(+1.825	277.2	(+1.825	(+1.825
	3	195.7 <sup>2</sup>	124.6	(+1.570	298.8	140.4	(+2.128	243.8 <sup>2</sup>	133.3	(+1.828	133.3	(+1.828	(+1.828
	4	103.9	83.7	(+1.277	147.3	68.0	(+2.166	125.2	75.0	(+1.669	75.0	(+1.669	(+1.669
	5	99.1	68.1	(+1.455	87.3	88.2	(-1.010	93.8	79.2	(+1.184	79.2	(+1.184	(+1.184
	6	102.4	68.4	(+1.497	77.3	92.7	(-1.199	91.1	81.8	(+1.113	81.8	(+1.113	(+1.113
	7	79.8	60.8	(+1.312	91.7	74.6	(+1.229	85.2	68.6	(+1.242	68.6	(+1.242	(+1.242
	8	72.1	60.3	(+1.195	74.3	69.5	(+1.069	73.1	65.4	(+1.117	65.4	(+1.117	(+1.117
Retention.....		11 rats	10 rats		12 rats	13 rats		23 rats	23 rats		23 rats		
	1	84.1	99.8	(-1.186	93.2	90.2	(+1.033	88.9	94.4	(+1.061	94.4	(+1.061	(+1.061
	2	53.2	60.1 <sup>3</sup>	(-1.129	56.8	51.9	(+1.094	55.1	55.3 <sup>3</sup>	(-1.003	55.3 <sup>3</sup>	(-1.003	(-1.003
	3	60.1	37.7	(+1.594	45.9	40.1	(+1.144	52.7	39.0	(+1.351	39.0	(+1.351	(+1.351
	4	48.0	29.9	(+1.605	90.3	47.3	(+1.909	70.1	39.7	(+1.765	39.7	(+1.765	(+1.765

<sup>1</sup> Omitting rat 1279: record for eighth day lost. <sup>2</sup> Omitting rat 1311: record for third day lost. <sup>3</sup> Omitting rat 1211: sick.

# TIME PER DAY RATIOS OF TESTS VS CONTROLS

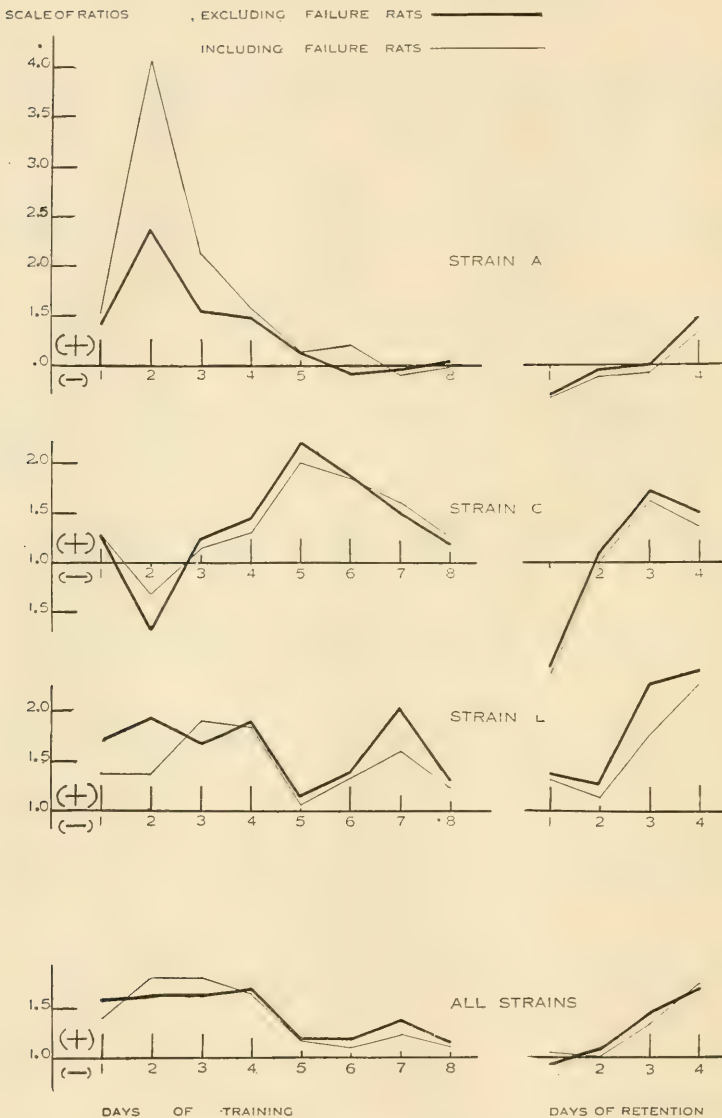


Fig. 4 Ratios of the time averages of the tests vs. controls on each day of training and retention. The base line represents equality, i.e., ratio of 1.000; points above the base line represent 'plus' ratios, those obtained when the test average was larger than, and so divided by, the control average; points below the base line represent 'minus' ratios, those obtained when the control average was larger than, and so divided by, the test average. Accordingly, points above the base line show that the tests took more time. The heavy lines are based on the 'completes' and 'incompletes'; light lines, upon the 'failures' as well. Units on the base lines are days: first series for training; the second series, retention; units on the vertical scale gives the size of the ratios.

Above the line are plotted the 'plus ratios' (those that indicate that the test average was higher than the control average); below the line are plotted the 'minus ratios' (those indicating that the control averages were higher than the tests). The heavy line connects the plotted ratios for the group of rats excluding the 'failure' rats; the light line includes the 'failure' rats. Obviously, the strong tendency in each strain is for the line connecting the ratios to lie well above the base line of equality; in other words, the test averages tend to be higher (that is, take more time) than the control averages.

The following tabulations are given in consideration of the size of the ratios:

*The number of ratios over 'plus' 1.500*

	MALES	FEMALES	MALES AND FEMALES
Excluding 'failure' rats .....	20	18	17
Including 'failure' rats .....	16	14	17

*The number of ratios over 'minus' 1.500*

	3	2	2
Excluding 'failure' rats .....	3	2	2
Including 'failure' rats .....	3	2	1

Excluding 'failure' rats, 42.5 per cent of the 'plus' ratios are above 1.500; 25 per cent of the 'minus' ratios are above 1.500; 17.5 per cent more of the 'plus' ratios are over 1.500. It is clear that besides being more frequent there is a larger proportion of high 'plus' ratios than high 'minus' ratios. This is brought out more plainly in the following graph (fig. 5), which gives the distributions of the ratios on each day for the sexes and strains separately (that is, not using ratios involving the repetition of data). The 'plus' ratios are shown in the solid line and the 'minus' ratios by the broken line. The mean of the 'plus' ratios is higher than the mean of the 'minus' ratios, but the difference has questionable statistical significance as tested by its probable error. (Average of 'plus' ratios = 1.685; average of 'minus' ratios = 1.478; difference  $0.207 \pm 0.09 = 2.30$  times the probable error.)



Unfortunately, probable errors can not be given for the groupings in which the strains or sexes are given separately, since the numbers are too small for the calculation of standard deviations; but such calculations have been made for the inclusive grouping of all strains and both sexes. In table 4 are given the differences between the test and control averages for each day of *training* and *retention*, both excluding and including the 'failure' rats. The probable errors of the differences and the differences in

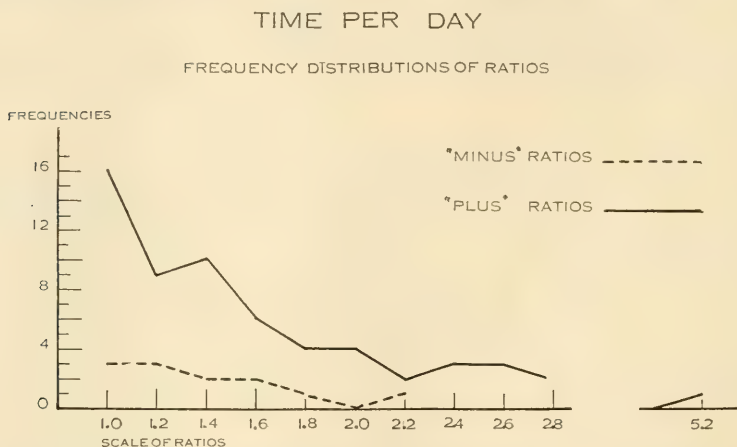


Fig. 5 Frequency distribution of the ratios of tests vs. controls, based on the averages of the time for each day with the sexes and strains treated separately (ratios given in table 3); the size of the ratios is shown on the base line; the vertical scale gives the numbers of ratios. The broken line gives 'minus' ratios (controls taking more time); the solid line gives 'plus' ratios (tests taking more time). 'Completes' and 'incompletes' are included, but not 'failures.'

terms of their probable errors (differences divided by their probable errors) indicate the statistical significance of the differences. The first four days of *training* and the last day of *retention* for the 'completes' and 'incompletes' show differences that are three or more times their probable errors. On the seventh day of *training* and the third day of *retention* the differences are only slightly below three times their probable errors (2.63 and 2.91 times). Adding the 'failures' gives the same results, with the exception that the difference on the third day of *training* falls a little below three times its probable error (2.51 times).

The fact of there being a series of twelve comparisons, eleven of which go in the same direction, has, in itself, a significance independent of the probable errors obtained in each case from the standard deviations. There is presented a series of twelve terms; each term may be plus or minus. If the sign is due to chance, there should be as many plus as minus terms in the

TABLE 4

*Showing the significance of the difference between the averages of the time of tests and controls on the different days of their training. These differences are taken from the averages of sexes and strains together in table 3. The plus sign indicates that the average of the test is higher than the corresponding average of the control. Since the differences in the first part of training are greater than three times their probable errors, the tests appear to take a significantly longer time in the maze in this part of training*

PERIOD	DAY	'COMPLETES' AND 'INCOMPLETES'			'COMPLETES' AND 'INCOMPLETES,' AND 'FAILURES'		
		Diff.	P. E.	Diff./P. E.	Diff.	P. E.	Diff./P. E.
Training.....	1	+686.0±143.3		4.79	+530.9±130.2		4.08
	2	+130.8± 39.3		3.33	+228.9± 56.7		4.03
	3	+ 62.2± 15.8		3.94	+110.5± 44.0		2.51
	4	+ 44.7± 9.8		4.54	+ 50.2± 14.3		3.50
	5	+ 14.2± 11.3		1.24	+ 14.6± 9.9		1.46
	6	+ 14.3± 11.0		1.30	+ 9.3± 9.5		0.98
	7	+ 24.3± 9.2		2.63	+ 16.6± 8.3		2.00
	8	+ 9.9± 8.4		1.16	+ 7.7± 7.3		1.06
Retention.....	1	- 5.2± 8.5		0.61	- 5.5± 8.3		0.66
	2	+ 3.8± 8.1		0.47	+ 0.2± 6.2		0.03
	3	+ 18.3± 6.2		2.91	+ 13.7± 5.5		2.50
	4	+ 27.5± 6.9		3.98	+ 30.4± 6.9		4.40

long run. The chances that eleven out of twelve should be plus on a chance basis would be the same as the frequency of eleven heads when twelve coins are tossed simultaneously; departures from equality as great as this may be expected to occur once in 157 trials, which corresponds to a little over four times the probable error.

When each day of *training* is considered by itself, the averages in the great majority of cases show that the tests took more

time than the controls in running the maze. In certain cases the tests took less time, but these differences tend to be smaller than the differences in the other direction. In the first half of the training period the differences are statistically significant.

Still further comparisons have been made, using the time for each successive trial instead of total time for each day. That is, averages of the first trials of all the rats in all the different combinations of sexes and strains have been calculated, and so for each of the thirty-six trials. This study gives results in full agreement with those obtained from the study of averages based on the total time for each day. The variability is naturally increased, but the number of points of comparison has been tripled; even without this evidence the conclusion is so unquestionable and the tables involved are so extensive that these summaries are not presented.

*c. Variability of the tests vs. controls, judged by time.* In table 5 are given the standard deviations of the averages for each rat for each of the six different groups of trials, when the males and females in all strains are put together. The tests have higher standard deviations in all groups of trials except in *retention* and, when the 'incompletes' and 'incompletes' and 'failures' are added, in the *second half of training*. The difference is more than three times the probable error of the difference in four of the six groups of trials when the 'completes' are alone; adding the 'incompletes' does not change this; but when the 'failure' rats are also added, none of the groups of trials give differences between the standard deviations that are significant; i.e., three times their probable error.

Table 6, which gives the standard deviations for each day, shows that the tests are significantly more variable on the first four days of *training* and on the fourth day of *retention*; with the 'failures' included, the tests are significantly more variable on the first, third, and fourth days of *training* and on the fourth day of *retention*. Although the differences in the standard deviations are not significant when the first twelve trials are taken together (*first half of training*) and the 'failures' included,





TABLE 6

*Standard deviations of the time spent on each day by each rat. The differences between the standard deviations are marked with a plus sign when the tests are more variable, and with a minus sign when the controls are more variable. The differences divided by their probable errors (Diff/PE) indicate that the tests are significantly more variable in the first half of training*

PERIOD	DAY	'COMPLETES' AND 'INCOMPLETES'				'COMPLETES,' 'INCOMPLETES,' AND 'FAILURES'			
		Tests	Controls	Diff.	Diff./P. E.	Tests	Controls	Diff.	Diff./P. E.
Training.....	1	929.9	478.5	+451.4±191.3	4.4	872.2	588.9	+283.3±92.0	3.1
	2	248.0	153.1	+94.9±27.8	3.4	354.2	296.4	+57.8±40.0	1.4
	3	96.1	63.6	+32.5±11.1	2.9	319.4	158.5	+160.9±31.1	5.2
	4	69.1	23.2	+45.9±6.9	6.6	112.2	36.7	+75.5±10.1	7.4
	5	50.1	67.0	-16.9±8.0	2.1	50.7	62.6	-11.9±7.0	1.7
	6	53.4	61.7	-8.3±7.8	1.0	50.2	58.5	-8.3±6.7	1.2
	7	51.2	45.1	+6.1±6.5	0.9	51.9	43.7	+8.2±5.8	1.4
	8	50.1	37.2	+12.9±5.9	2.2	46.3	36.8	+9.5±5.2	1.8
Retention.....	1	38.1	40.6	-2.5±6.0	0.4	39.9	43.9	-4.0±5.9	0.6
	2	35.1	38.8	-3.7±5.7	0.6	33.2	36.8	-3.6±4.9	0.7
	3	30.0	27.6	+2.4±4.4	0.5	28.8	26.7	+2.0±3.8	0.5
	4	39.1	22.1	+17.0±4.8	3.5	43.4	22.8	+20.6±1.8	4.2

significant differences in the variability are found when the trials are separated into groups of three. It may be concluded, then, that there is a difference in the variability of the tests and controls in the first half of training, although when the 'failure' rats are included this is hidden if all the twelve trials are taken together.

*d. What is the probability that the test data and control data for time are not random samples from the same population?*<sup>2</sup> It is believed that sufficient evidence has been presented to remove the slightest doubt from the conclusion that the tests as a group took more time in learning the maze than did the controls. So the presentation of the following method of comparing the time data of the tests and controls is of more interest from a methodological standpoint than from the standpoint of the findings, which, it may be stated in advance, fully substantiate the above conclusions.

Although the  $\chi^2$  test is usually employed to measure the goodness of fit of a theoretical and an empirical curve, it also affords a method of measuring how poorly two curves agree; it measures the probability that two curves, differing as much as those in question, will occur as the result of random sampling from a single population. The following formula given by Pearson (*Biometrika*, '11, p. 250) has been used:

$$\chi^2 = \Sigma \left\{ \frac{NN' \left( \frac{f}{N} - \frac{f'}{N'} \right)^2}{f + f'} \right\}$$

in which  $N$  = numbers in one sample;  $N'$  = numbers in the other sample;  $f$  = frequency of a class in one sample,  $f'$  = the frequency of a class in the other sample.

If the  $\chi^2$  test indicates that such differences as are found may be expected to occur more often than once in twenty times (once in twenty times corresponds to three times the probable error), the curves could not be considered significantly different;

<sup>2</sup> We are pleased to acknowledge the large part our colleague, Prof. H. D. Fish, has contributed in working out the methods used in this section.

but if it shows that curves as different may be expected to occur less often than once in twenty times, they would be considered significantly different.

The number of rats is too small to permit the calculation of  $\chi^2$  from curves based on the averages of each rat. But by using every trial of every rat, enough numbers are obtained. However, the actual time at the beginning and the end of training is so very different that it would not be possible to make satisfactory distribution curves by seriating these directly. For this reason ratios were substituted for the actual trials. A standard smooth curve was established by interpolation from the means of the control rats for each successive trial in *training*. For each trial of each rat (tests and controls) the ratio to the corresponding point on this assumed standard curve was calculated. In obtaining these ratios the smaller number was always divided into the larger; when the actual time was longer than the standard, the ratio was called 'plus,' when shorter, 'minus.' These ratios, tests and controls separately, were next seriated upon a scale with equality (ratio = 1.00) as the central point and 'plus' ratios of increasing size extending to the right, 'minus' ratios of increasing size extending to the left. Figure 6 shows the distribution curves for these ratios grouped in classes of 1.725 in width, and also into classes 2.625 wide. The solid line represents the controls, the broken line the tests. In one pair of curves  $\chi^2 = 58.5$ ; on the other hand,  $\chi^2 = 44.5$ . The value for P given in biometrika tables (p. 25, table XII) for both these values of  $\chi^2$  and the number of classes in each curve is 0.000,000. In other words, the odds against these pairs of curves being random samples from the same population are at least greater than 1,000,000 to 1.

*e. Conclusions based on the time data.* The data on time have been summarized in various ways with three groupings of the rats: 1) 'completes,' 2) 'completes' and 'incompletes,' 3) 'completes,' 'incompletes,' and 'failures;' averages per rat for the different periods of the training, averages per rat for each day by itself, males and females separately and together, the three strains grouped separately and together; in each method of

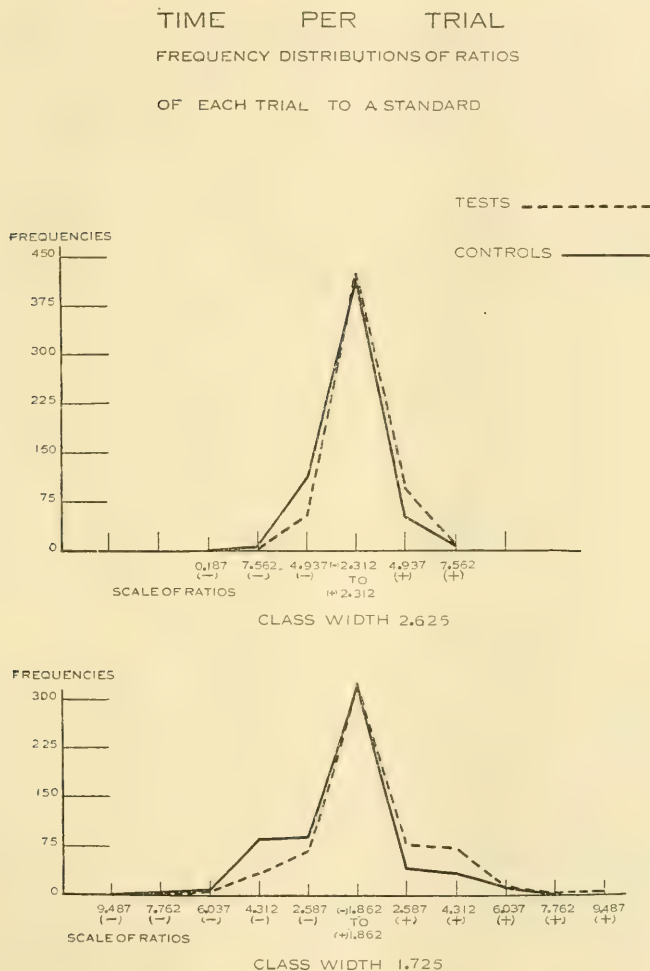


Fig. 6 Distributions of the ratios obtained by dividing each time record into, or by, the assumed standard for the corresponding trial in the training; 'plus' ratios, those at the right of the central point, were obtained when the record was larger than the standard, and so divided by the standard; 'minus' ratios, those at the left, were obtained when the record was smaller than the standard, and so divided into the standard. These ratios are grouped into classes of two widths, 2.625 and 1.725 wide, respectively. The numbers on the base lines are the outer limits of the ratios included in the classes; the vertical scale gives the numbers of ratios in each class. Broken lines show the tests; solid lines, the controls. These distributions have been used for the application of the  $\chi^2$  test.



summarizing the tests and controls have been compared. Standard deviations and probable errors have been calculated for all groupings that included large enough numbers; a special set of ratios to an assumed standard has been obtained for the purpose of applying the  $\chi^2$  test to the frequency distributions of the test and control data. Every method of treating the time data leads to the same conclusion, namely, that the test rats as a group differ from the controls; this difference is more apparent in the first half of *training* while the learning was rapid, but even in the last half of *training*, after there was very little general improvement a similar though smaller difference is found. Tested by probable errors, significant differences are found between the averages involving the first half of *training* and the fourth day of *retention*; the distributions of all the data for tests and controls, compared by means of the  $\chi^2$  test, are found to differ more widely than could be explained by random sampling.

3. *Comparison of the test and control rats on the basis of the distance covered in running each trial*

a. *Distance: averages for different groups of trials.* As explained in the first section, the data on distance were obtained from camera-lucida drawings of the course of each trial of each rat. As the rat went through the alleys of the maze, its image reflected upon the record sheet was followed with a pencil, thus making an accurate and lasting record of just where the rat went. These pencil lines were measured by means of a map measurer, or chartometer. Figure 7 reproduces a record sheet; it bears an inverted plan of the maze. These sheets are about 1/13th the size of the maze itself. In the early trials, when the rats were going long distances, several sheets were used to record one run. The data presented are in terms of the actual lengths in centimeters of the pencil lines that appear on the record sheets; accordingly, all the distances are 1/13th of the actual distances covered by the rats, but the relative difference between the tests and controls is the same as though the actual distances had been used. Leaving the data in the reduced form has saved the labor of transforming each record into the actual distance, as well as of handling much larger numbers in the summarizing.

The distance data have been summarized for the 'completes' and 'incompletes' without giving 'completes' alone or including the 'failures,' for reasons given on page 217; otherwise, the same methods have been employed as for the time data. Table 7

No. 1211

Time :08.2 sec.  
Distance .37 cm.



Fig. 7 Record sheet four-fifths actual size. This shows an inverted plan of the maze as reflected upon the sheet; the rats were started at *S* and found food in *F*. The maze itself is 5 feet in diameter.

gives the averages for each of the six groupings of the trials (averages of the averages for each rat). The following groups of the rats have been used: sexes separate and strains separate; sexes together and strains separate; sexes separate and strains together; sexes together and strains together. The averages

TABLE 7

Distance per trial in centimeters for six groupings of the trials; numbers in the table are averages of the averages for each rat. Just the 'completes' and 'incompletes' are used here. The ratio of the averages when 'plus' indicates that the test average is larger and has been divided by the control average. The great predominance of 'plus' ratios shows that the tests covered more distance in whatever way the trials are grouped

STRAIN	PERIOD	MALES			FEMALES			MALES AND FEMALES		
		Tests	Controls	Ratios	Tests	Controls	Ratios	Tests	Controls	Ratios
A	First half training	5 rats 256.2	3 rats 159.5	(+1.663)	3 rats 173.9	7 rats 160.3	(+1.085)	8 rats 239.1	10 rats 160.1	(+1.493)
	Second half training	112.8	77.2	(+1.464)	89.2	107.4	(-1.204)	104.0	98.2	(+1.059)
	Omitting first day	136.4	94.9	(+1.437)	97.8	105.6	(-1.079)	121.9	102.4	(+1.190)
	All training	189.0	118.2	(+1.599)	134.0	133.8	(+1.002)	173.3	129.1	(+1.342)
	Retention	92.4	75.4	(+1.225)	69.5	83.4	(-1.200)	80.9	80.0	(+1.011)
C	Training and retention	144.1	103.9	(+1.386)	112.0	116.0	(-1.043)	131.2	111.4	(+1.179)
	First half training	4 rats 185.5	3 rats 132.8	(+1.344)	1 rat 264.0	2 rats 154.8	(+1.706)	5 rats 195.6	5 rats 141.6	(+1.381)
	Second half training	79.6	47.6	(+1.672)	99.4	42.2	(+2.355)	81.4	45.5	(+1.789)
	Omitting first day	102.7	60.3	(+1.703)	127.3	71.0	(+1.791)	107.6	64.6	(+1.666)
	All training	127.8	90.2	(+1.417)	181.7	98.5	(+1.844)	138.5	93.5	(+1.481)
L	Retention	58.7	48.4	(+1.212)	80.0	52.2	(+1.532)	64.0	50.3	(+1.272)
	Training and retention	109.1	79.7	(+1.368)	147.8	83.2	(+1.780)	118.8	81.0	(+1.466)
	First half training	7 rats 210.3	4 rats 184.1	(+1.142)	5 rats 183.4	6 rats 142.4	(+1.287)	11 rats 198.1	10 rats 159.1	(+1.245)
	Second half training	97.2	81.5	(+1.192)	74.4	67.7	(+1.098)	87.7	73.2	(+1.198)
	Omitting first day	123.2	90.4	(+1.363)	91.4	75.5	(+1.210)	108.8	81.5	(+1.335)
All	All training	156.9	132.8	(+1.181)	128.9	100.0	(+1.289)	144.2	113.1	(+1.274)
	Retention	60.7	52.7	(+1.158)	72.6	56.2	(+1.291)	67.3	55.3	(+1.216)
	Training and retention	122.9	98.0	(+1.254)	110.1	85.4	(+1.289)	114.9	88.6	(+1.297)
	First half training	16 rats 220.1	10 rats 161.3	(+1.364)	9 rats 191.1	15 rats 152.4	(+1.254)	25 rats 210.1	25 rats 155.9	(+1.348)
	Second half training	97.0	70.0	(+1.385)	82.1	82.8	(-1.008)	91.6	77.7	(+1.179)
	Omitting first day	122.1	82.7	(+1.476)	97.5	88.9	(+1.097)	112.9	86.5	(+1.305)
	All training	159.8	115.6	(+1.382)	136.8	115.5	(+1.184)	151.8	115.6	(+1.313)
	Retention	96.6	61.1	(+1.139)	72.4	64.6	(+1.129)	70.9	63.3	(+1.120)
	Training and retention	125.3	93.3	(+1.342)	115.3	95.5	(+1.207)	120.6	94.6	(+1.274)

of the tests and controls are compared by the ratios of one to the other and not by their differences; a 'plus' ratio indicates that the test average is higher and has been divided by the control average; a 'minus' ratio indicates that the control average is higher and has been divided by the test average.

In the whole table there are sixty-seven 'plus' ratios against five 'minus' ratios. The highest 'minus' ratio is 1.204; forty-nine of the 'plus' ratios are higher than this. The males give 'plus' ratios in all cases. The females give the five 'minus' ratios; strain A seems responsible for these. When the sexes

TABLE 8

*Showing the significance of the differences between the distance averages, based on the averages of each rat for the different groupings of the trials. The differences are taken from the averages for sexes and strains together in table 7. Plus signs indicate that the averages of the tests are higher than the controls; in five of the six groupings the differences are probably significant, indicating that the distances covered by the tests are significantly greater*

PERIOD	DIFF.	DIFF./P. E.
First half training . . . . .	+54.2±9.2	5.9
Second half training . . . . .	+13.9±5.1	2.7
Omitting first day . . . . .	+26.4±5.3	5.0
All training . . . . .	+36.2±6.2	5.8
Retention . . . . .	+ 7.6±4.2	1.8
Training and retention . . . . .	+26.0±5.2	5.0

are combined all the ratios become plus. In table 8 are given the differences between the test and control averages which include all the rats of both sexes and all strains, the probable errors of the differences, and the differences in terms of their probable errors.

In four of the six groups of trials the differences are well above three times their probable errors (5.9, 5.0, 5.8, and 5.0 times, respectively). For the *second half of training* the difference is only slightly below three times its probable error (2.7 times), but for *retention* the difference is only 1.8 times its probable error. For distance, as for time, the difference in favor of the controls is fully significant during the initial period of rapid



learning, while in later trials the difference is reduced. Figure 8 gives the distributions of the averages of the individual rats for the six groups of trials. There appears a strong tendency for the test rats to lie further up the scale (to the right) than the controls. This is less marked in the *second half of training*; and in *retention* there is very little difference between the distributions of the tests and controls. The averages of the distance for each rat for each of the groups of trials from which the preceding summaries have been made are given in table B in the appendix.

*b. Distance: averages for each day.* The next step in the averages of the distance data is the comparison of the tests and controls on each of the eight days of *training* and on the four days of *retention*. In table 9 the averages are arranged in the same combination of sexes and strains as before, and then compared by their ratios; 'plus' ratios indicate that the tests covered more distance than the controls. The averages for each day, when the sexes and strains are put together, are shown by the curves in figure 9; in figure 10 are represented graphically the ratios of the tests vs. controls for the males and females together and the strains separately as well as together. Obviously, the tests covered more ground in the great majority of cases. The females in strain A show the reverse, but there can be no doubt that this is not a general sex difference nor a strain difference, since the females in the other two strains and the males in all three strains do show that the controls covered shorter distances on the average than did the tests. There is a tendency for the first day of *retention* to show the tests covering less distance, but it will be noticed that this is true of two of the strains, not of the third (L). The real interest lies in the table (10) where the significance of the differences may be found. This table is based on all the males and females in all strains together. It appears that on the first, third, and fourth days of *training* and on the fourth day of *retention* the differences between the averages of the tests and controls are more than three times their probable errors; on the second and sixth days of *training* the differences are only very slightly below three times their probable errors

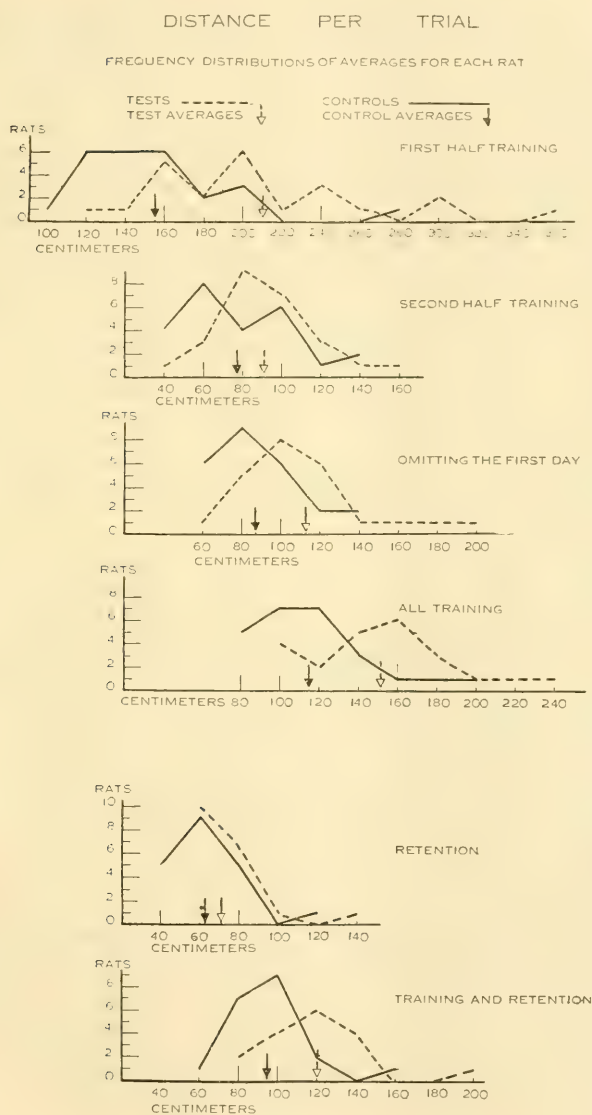


Fig. 8 Frequency distributions of the averages for each rat for distance per trial, based on six groupings of the trials. Broken lines give the tests; solid lines, the controls; units on the base line are centimeters; on the vertical scale, numbers of individuals; males and females in all strains are included, using the 'completes' and 'incompletes,' but not the 'failures.'

TABLE 9

*Distance in centimeters for each day of the training separately; averages of the total distance (as measured on the record sheets) covered on each day by each rat; the comparisons are made for the 'completes' and 'incompletes' together. The averages are compared by ratios: a 'plus' ratio indicates that the test average is higher and has been divided by the control average, while a 'minus' ratio indicates that the control average is higher and has been divided by the test average. With the exception of the females in strain A, most of the comparisons show that the tests covered more distance on each day than did the controls*

	DAY	MALES			FEMALES			MALES AND FEMALES		
		Tests		Ratios	Tests		Ratios	Tests	Controls	Ratios
		5 rats	3 rats		3 rats	7 rats		8 rats	10 rats	
Strain A	1	1673.4	845.6	(+1.978	987.5 <sup>1</sup>	1034.0	(-1.047	1477.4 <sup>1</sup>	977.5	(+1.511
	2	718.8	483.0	(+1.488	372.3	338.9	(+1.098	588.9	382.1	(+1.541
	3	448.4	368.3	(+1.217	373.6	379.9	(+1.334	420.4	306.4	(+1.372
	4	342.4	217.6	(+1.573	237.3	271.4	(-1.143	303.0	255.3	(+1.186
	5	437.6	247.3	(+1.769	271.3	365.8	(-1.348	375.2	330.3	(+1.135
	6	393.4	230.3	(+1.708	264.3	322.7	(-1.220	345.0	295.0	(+1.169
	7	258.6	229.6	(+1.126	278.6	359.8	(-1.291	266.1	320.8	(-1.205
	8	265.0	217.3	(+1.219	257.0	307.3	(-1.195	267.4	280.3	(-1.048
Retention	1	3 rats	3 rats		3 rats	4 rats		6 rats	7 rats	
	2	234.6	296.0	(-1.261	244.3	297.7	(-1.218	239.5	297.0	(-1.240
	3	318.3	244.0	(+1.304	186.0	209.5	(-1.126	252.5	224.2	(+1.126
	4	264.3	189.3	(+1.396	185.6	259.7	(-1.399	225.0	229.5	(-1.020
Strain C	1	292.6	176.3	(+1.659	218.3	234.2	(-1.072	255.5	209.4	(+1.220
	2									
	3									
	4									
Training	1	4 rats	3 rats		1 rat	2 rats		5 rats	5 rats	
	2	1212.0	900.0	(+1.346	1689.0	873.5	(+1.933	1307.2	889.4	(+1.469
	3	373.0	331.0	(+1.126	702.0	606.5	(+1.157	438.8	441.2	(-1.005
	4	315.0	188.3	(+1.672	447.0	223.5	(+2.000	341.4	202.4	(+1.686





TABLE 9—Continued

	DAY	MALES			FEMALES			MALES AND FEMALES		
		Tests	Controls	Ratios	Tests	Controls	Ratios	Tests	Controls	Ratios
<i>All strains</i>		16 rats	10 rats	(+).1.387	9 rats	15 rats	(+).1.224	25 rats	25 rats	(+).1.358
	1	1441.5	1038.9	(+).1.356	1191.9 <sup>1</sup>	973.5	(+).1.075	1358.3 <sup>1</sup>	999.7	(+).1.244
	2	510.4	376.4	(+).1.166	415.8	386.7	(+).1.452	473.6	382.7	(+).1.321
	3	356.2	305.4	(+).1.304	357.2	245.9	(+).1.300	356.5 <sup>3</sup>	269.7	(+).1.292
	4	281.1	215.5	(-).1.390	290.3	223.2	(+).1.007	284.4	220.1	(+).1.200
	5	322.5	232.0	(+).1.454	267.5	265.5	(-).1.043	302.7	252.1	(+).1.223
	6	325.4	223.7	(+).1.360	243.3	253.9	(+).1.004	295.8	241.8	(+).1.144
	7	269.6	198.1	(+).1.322	252.9	251.8	(-).1.114	263.6	230.3	(+).1.066
<i>Training . . . . .</i>	8	247.1	186.8	(-).1.579	222.2	247.6	(+).1.336	238.2	223.3	(+).1.377
		10 rats	8 rats	(-).1.107	9 rats	12 rats	(+).1.007	19 rats	20 rats	(-).1.044
	1	225.9	250.2	(+).1.204	238.6	236.9	(+).1.142	231.9	242.2	(+).1.167
	2	220.7	183.3 <sup>2</sup>	(+).1.434	214.1	187.4	(+).1.062	217.0	185.9 <sup>2</sup>	(+).1.207
	3	223.3	155.7	(-).1.579	173.8	163.5	(+).1.336	199.7	165.4	(+).1.377
	4	207.2	131.2	(-).1.579	243.4	182.1	(+).1.336	224.3	162.8	(+).1.377
		10 rats	8 rats	(-).1.107	9 rats	12 rats	(+).1.007	19 rats	20 rats	(-).1.044
	1	225.9	250.2	(+).1.204	238.6	236.9	(+).1.142	231.9	242.2	(+).1.167
<i>Retention . . . . .</i>	2	220.7	183.3 <sup>2</sup>	(+).1.434	214.1	187.4	(+).1.062	217.0	185.9 <sup>2</sup>	(+).1.207
	3	223.3	155.7	(-).1.579	173.8	163.5	(+).1.336	199.7	165.4	(+).1.377
	4	207.2	131.2	(-).1.579	243.4	182.1	(+).1.336	224.3	162.8	(+).1.377
		10 rats	8 rats	(-).1.107	9 rats	12 rats	(+).1.007	19 rats	20 rats	(-).1.044

<sup>1</sup> Omitting rat 1279; record lost. <sup>2</sup> Omitting rat 1211; sick. <sup>3</sup> Omitting rat 1311; record lost.

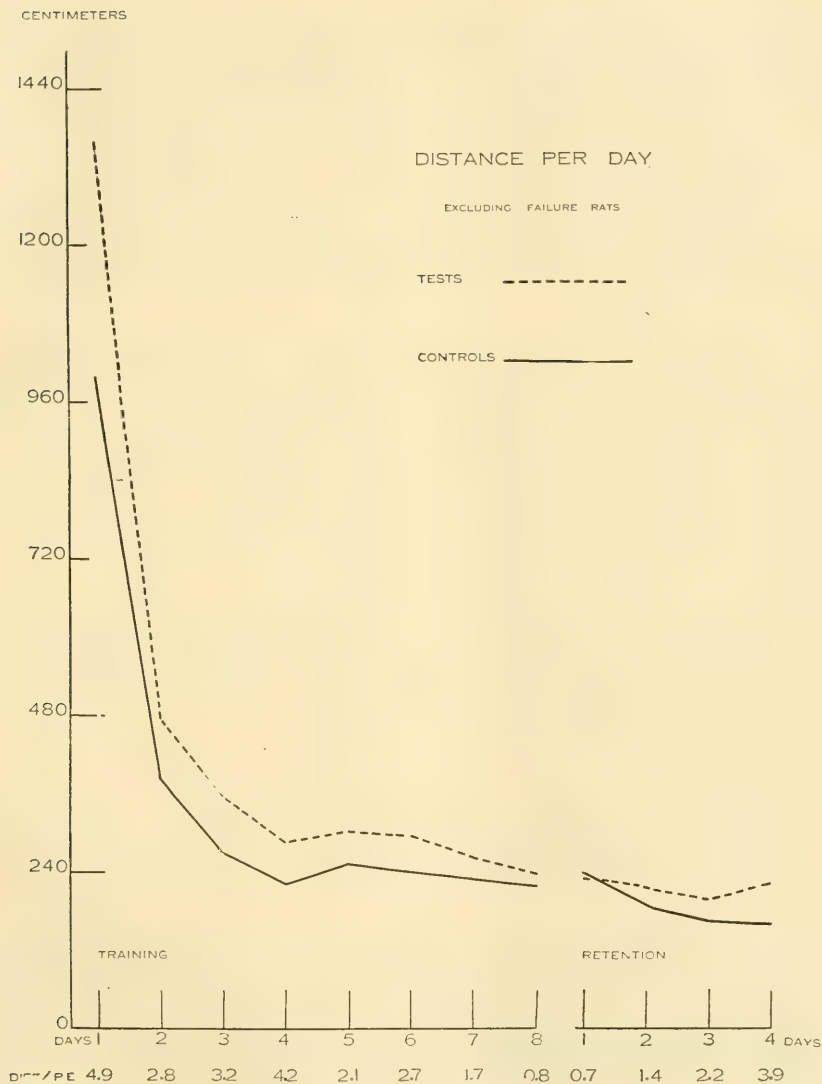


Fig. 9 Learning curve based on distance; averages per trial on each day of *training* and *retention*. The broken lines are tests; the solid lines, controls. Units on the base line are days, units on the vertical scale are centimeters. The first series of days give *training*; the second series, *retention*. Males and females in all strains are put together in all averages. Based on the 'completes' and 'incompletes.'

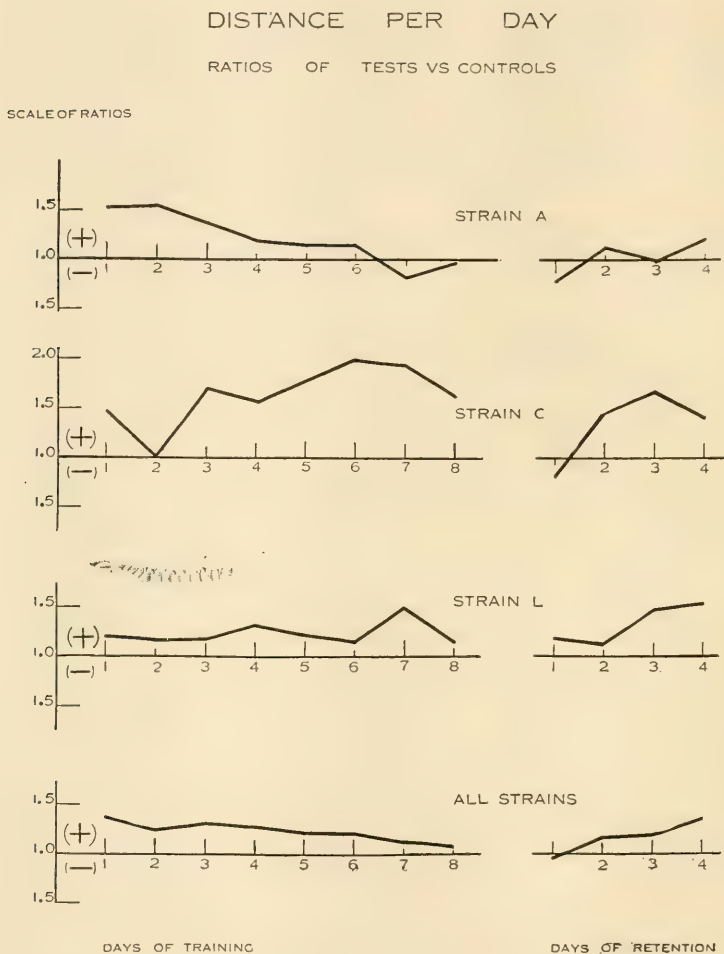


Fig. 10 Ratios of the distance averages of tests vs. controls on each day of training and retention. The base line represents equality of the test and control averages; that is, ratios of 1.000; points above the base line represent 'plus' ratios, obtained when the test average was larger and so divided by the control average; points below the base line represent 'minus' ratios, obtained when the control average was larger and so divided by the test average. Accordingly, points above the line show that the tests covered more distance than the controls. 'Completes' and 'incompletes' have been used, males and females together; vertical scale gives the size of the ratios; horizontal scale, days; the first series of days gives *training*; the second series, *retention*.

(2.8 and 2.7 times). There are, then, six days on which the differences are probably significant, indicating that the tests went further than the controls, and there are no significant differences indicating the reverse.

Moreover that eleven out of twelve days should have the same sign has a significance quite apart from the probable errors on the individual days. Departures from equality as great as this may be expected in one out of 157 times if there is no real

TABLE 10

*Showing the significance of the differences between the distance averages, based on the total distance covered by each rat on each day. These differences are taken from the averages for sexes and strains together in table 9. The plus sign indicates that the average of the test is higher than that of the control. In the first half of training the differences are either close to three times their probable errors or higher, so they may be considered significant*

PERIOD	DAY	DIFF.	DIFF./P. E.
Training.....	1	+358.6±73.1	4.9
	2	+93.6±33.6	2.8
	3	+86.8±27.0	3.2
	4	+64.3±15.4	4.2
	5	+50.6±23.9	2.1
	6	+54.0±19.9	2.7
	7	+33.3±19.3	1.7
	8	+14.9±19.5	0.8
Retention.....	1	-10.3±14.9	0.7
	2	+31.1±21.8	1.4
	3	+34.3±15.7	2.2
	4	+61.5±15.8	3.9

difference between the tests and controls; but this is a smaller probability than when a difference is over four times its probable error.

A better idea of the differences between the 'plus' and 'minus' ratios in table 9 is given by the graphs in figure 11. In these curves the frequency distribution of the 'plus' ratios is given in the solid line, of the 'minus' ratios in the broken line. These distributions include only those ratios that do not involve repetition of any primary data, namely, for males and females



separately in the separate strains. The fifty-seven 'plus' ratios have an average of 1.503; the fifteen 'minus' ratios have an average of 1.203. The difference between these averages ( $0.300 \pm 0.048$ ) is 6.2 times its probable error. Besides a larger number of the ratios being 'plus,' it appears that the average size of the 'plus' ratios is significantly greater than the average size of the 'minus' ratios.

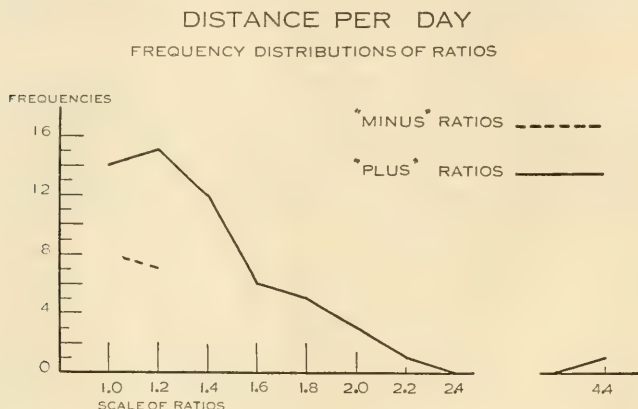


Fig. 11 Frequency distributions of the ratios of tests vs. controls, based on the averages of the distance for each day, with sexes and strains treated separately; the ratios are taken from table 9. The size of the ratios is shown on the base line, the vertical scale gives the frequencies; the broken line shows the 'minus' ratios (controls taking more time); the solid line, the 'plus' ratios (tests taking more time); 'completes' and 'incompletes' included, but not 'failures.'

*c. Variability of the tests vs. controls as judged by distance.* Table 11 gives the standard deviations for the averages for each rat for each of the six groupings of the trials (see the distributions of the averages in figure 8). For the *first half of training, omitting the first day, training, and training and retention*, the standard deviations for the tests are higher; for the *second half training and retention* the standard deviations for the controls are slightly higher. This shows an agreement between the size of the means and the standard deviations, for it is in these last two groups of trials that the control averages are not significant. However, the differences between these standard deviations are in no group

of trials surely significant; the nearest approach to three times the probable error is 2.77 times, for *omitting the first day*; then comes the *first half of training, training and retention*, and *training* with differences 2.49, 2.09, and 1.89 times their probable errors; for the other two groups of trials the differences are less than half as large as their probable errors. When the standard deviation of the averages for each day are calculated (table 12) the tests are proved the more variable on the first six days of *training* and on the second and fourth days of *retention*; but

TABLE 11

*Standard deviations of the distance averages for each rat when all strains and both sexes are put together for the six groupings of the trials, calculated from the data given in table B in the appendix. The differences, their probable errors, and the differences divided by their probable errors are given. Plus signs indicate that the standard deviations of the tests are greater than those of the controls; minus signs, that the standard deviations of the controls are larger. Although there are no fully significant differences, the differences that are over twice their probable errors are all in the direction of greater variability for the tests*

PERIOD	TESTS	CONTROLS	DIFF.	DIFF./P. E.
First half training . . . . .	54.24	38.08	+16.16±6.40	2.49
Second half training . . . . .	26.14	27.62	- 1.48±3.62	0.41
Omitting first day . . . . .	32.50	22.01	+10.49±3.79	2.77
All training . . . . .	35.66	27.37	+ 8.29±4.39	1.89
Retention . . . . .	19.35	19.78 <sup>1</sup>	- 0.43±3.02	0.14
Training and retention . . . . .	26.46	18.81	+ 7.65±3.65	2.09

<sup>1</sup> Omitting rat 1211.

when compared with their probable errors, the only significant difference is found on the second day of retention. On the seventh day of *training* the controls are significantly more variable. There does not seem to be any general difference in the variability of the averages of the tests and controls based on distance, although five of the comparisons show greater variability for the tests by differences twice their probable errors; there are only two significant differences, one showing the controls more variable, the other showing the tests more variable.

Averages for each of the thirty-six trials in *training* and *retention* have been obtained and the tests and controls compared on

the basis of each trial by itself. The averages for all the various groupings of strains and sexes have been computed; the results are in full accord with those obtained when the averages for each day were compared. They are not presented because the additional evidence they would offer does not seem needed.

*d. What is the probability that the test data and control data for distance are not random samples of the same population?* From the control averages of each of the twenty-four successive trials

TABLE 12

*Standard deviations of the averages for each rat when all strains and both sexes are put together for each day of the training. Conventions as in table 11*

PERIOD	DAY	TESTS	CONTROLS	DIFF. P. E.	DIFF./P. E.
Training.....	1	422.0	330.0	+92.0±51.8	1.7
	2	200.9	148.7	+52.2±23.8	2.2
	3	144.0	136.3	+ 7.7±19.0	0.4
	4	83.7	77.6	+ 6.1± 1.9	0.5
	5	126.4	124.4	+ 2.0±16.8	0.1
	6	112.2	96.7	+15.5±14.1	1.1
	7	76.1	121.7	-45.6±13.6	3.3
	8	102.3	103.6	- 1.3±13.8	0.1
Retention.....	1	59.2	77.9	-18.7±10.5	1.8
	2	127.0	64.4 <sup>1</sup>	+62.6±15.6	4.0
	3	71.0	75.1	- 4.1±11.1	0.3
	4	80.4	65.4	+15.0±11.2	1.3

<sup>1</sup> Omitting rat 1211.

in *training* a curve was smoothed by interpolation; this was taken as the standard of comparison in computing a ratio for each trial of each rat. The distribution of these ratios above and below the central point of 1.000, or unity, is shown in figure 12. Ratios at the left of the center are from trials less (shorter distance) than the assumed standard; ratios at the right of the center are from trials that were longer than the assumed standard. The ratios for the tests are shown by the broken line; the ratios for the controls by the solid line. In the first graph the ratios are classified in classes 1.699 in width; in the second graph the

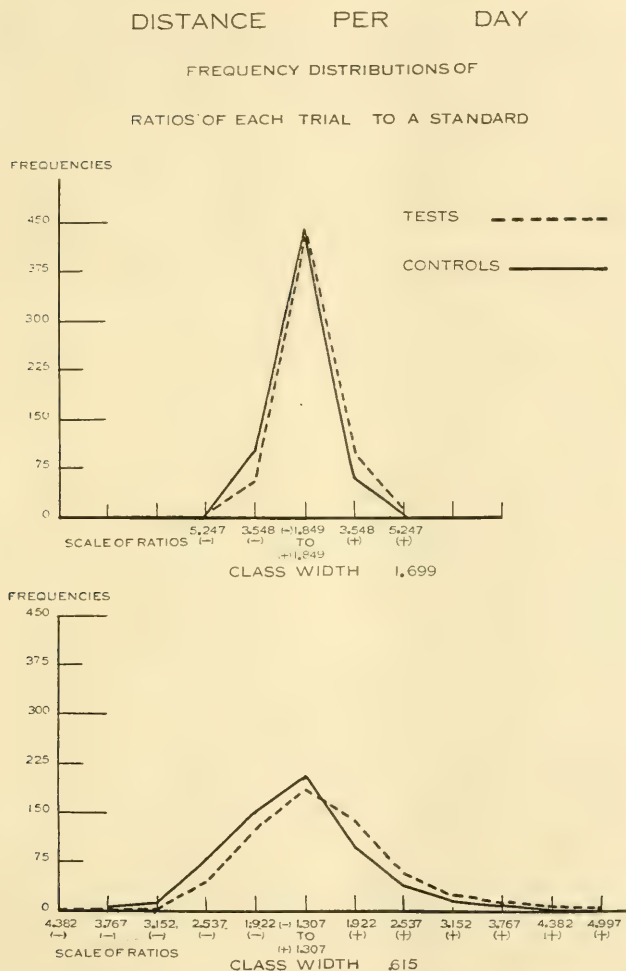


Fig. 12 Frequency distributions used for the application of the  $\chi^2$  test. Distributions of the ratios obtained by dividing each distance record into, or by an assumed standard for the corresponding trial. 'Plus' ratios, those at the right of the central point, were obtained when the record was larger than the standard; 'minus' ratios, those at the left of the center, were obtained when the record was smaller than the standard. These ratios are grouped in two ways: into classes 1.699 and 0.615 wide. The numbers on the base line are the extreme ratios included in the respective classes; the vertical scale gives the number of ratios in each class. Broken lines are for the test ratios, solid lines for the control ratios.



classes are 0.615 in width. The  $\chi^2$  obtained from the two pairs of curves are 66.49 and 84.78, both of which give values for P of 0.000,000. In other words, the odds against curves as different as these occurring as random samples of the same population are at least over 1,000,000 to 1. As far as this statistical test is concerned, the distributions for tests and controls are proved to be different.

*e. Summary of results from the distance data.* When the test and control rats are compared on the basis of the distance covered in running their trials on the maze, the tests are found to go further. This has been shown in the summaries of the rats in various groupings according to the sex and strain, when the averages of each rat for six different groups of its trials are used as the basis, and when the averages of the total distances covered on each of the twelve days are used as the basis of comparison. Tested by their probable errors, significant differences are found for each period excepting the *second half of training* and the first three days of *retention*.

Apparently both sets of rats can completely master the maze, but the tests take more trials to cut down the excess distance than do the controls. It is a matter of the number of trials, then, before all the excess distance of both sets of rats is eliminated. However, when all the trials of the tests and of the controls in the form of ratios to the corresponding points in an assumed normal standard curve are classified in frequency distributions, the  $\chi^2$  test indicates that there is a real difference in the distributions of the test and control ratios. All these results are in close agreement with those from the time data. The two sets of data are derived from the same movements of the same rats, but they differ in the mechanics of their origins; time from readings of the stop-watch operated synchronously with the rats' departures and arrivals, and distance from the readings of the chartometer after tracing the pencil lines on the record sheets.

*f. Correlation between time and distance data.* To test mathematically the similarity between the time and distance criteria, correlation coefficients have been calculated from the data of

the group of 'completes.' For the test rats the correlation coefficient of  $+0.9537 \pm 0.0028$  was found; for the controls,  $+0.9607 \pm 0.0023$ , a difference of  $0.0070 \pm 0.0036$  which is 1.9 times its probable error. This indicates that the test and control rats agree in showing that the two criteria of time and distance are very closely related; if a rat makes a very poor time record, it generally has a poor distance record, and so if it makes a good time record the distance record is generally good. But, although the correlation coefficients take both these good and poor records into account, there is a difference in the relation in the two cases. It would be impossible for a very short time record to involve a long distance, but a very long time record may give a relatively short distance record; the rat may go very slowly along the correct path, or it may lie down. This means that in one half of the correlation table points may be located almost anywhere, but in the other half they are greatly restricted. In table 13 is given the part of the combined correlation tables for tests and controls that excludes trials taking more than five minutes. In the terms of the correlation coefficients the time and distance criteria are so nearly alike that they could be considered the same. This similarity is not great enough, however, to remove all the differences in the details of the summaries based on the two criteria separately, but as far as the comparison of the tests and controls is concerned in the present problem, the two criteria give the same general conclusions.

We have presented this mathematical proof as well as the empirical demonstration of the similarity of the results given by the two criteria not because there seemed any doubt as to the validity of either method, but to make it perfectly clear to all that the results are entirely independent of the method of treatment. For this same reason we have made the comparisons between the same groups of rats upon still another set of data derived equally from time and distance.

*Correlation table of the time and distance data for all trials of the tests and controls together, that took five minutes or less. The numbers on the scales are the upper limits of the classes: at the top, distance in centimeters; at the left side, time in seconds. The correlation coefficients quoted in the text are based on all the trials, but with the tests and controls in separate tables*

[illegible]

#### 4. Comparison of the test and control rats on the basis of the rate of running (speed)

The relationship between the time and distance records for each rat has been used as the basis of another comparison of the tests and controls, namely, the average speed of running, in terms of the number of centimeters covered per second (as measured on the record sheets). For each rat the distance covered on each day has been divided by the time spent on the same day.

TABLE 14

*Average speed (centimeters per second) for training and retention. Plus signs indicate that the speed of the controls was greater than that of the tests. The controls in two strains had higher speed; the tests in one strain had higher speed*

STRAIN	PERIOD	MALES			FEMALES		
		Tests	Controls	Ratio	Tests	Controls	Ratio
A	Training	3.187	3.549	(+)1.113	2.342	3.084	(+)1.316
	Retention	4.010	3.806	(-)1.053	2.955	3.772	(+)1.276
C	Training	4.020	3.525	(-)1.140	3.883	3.593	(-)1.080
	Retention	4.840	3.787	(-)1.278	4.250	3.951	(-)1.075
L	Training	2.549	2.848	(+)1.117	2.579	3.034	(+)1.176
	Retention	3.166	4.170	(+)1.317	3.571	4.300	(+)1.204
All	Training	3.258	3.262	(+)1.001	2.645	3.144	(+)1.188
	Retention	3.921	3.890	(-)1.007	3.441	4.066	(+)1.164

From these figures the averages for *training* and *retention* for the various combinations of sexes and strains have been obtained for the 'completes' (table 14). In *training* the speed of the tests is less than that of the controls both for the males and females in strains A and L; but in strain C the males, as well as the females, give the averages for the tests higher than the controls. The same general relations hold for *retention*, the exception being the males in strain A, which give a higher average for the tests. Putting sexes and strains together gives higher averages for the controls in both *training* and *retention* (table 15), but in neither case is the difference significant. In *training* the difference is



only half as large as its probable error (0.44 times); in *retention* the difference is about twice as large as its probable error (2.07 times).

No claim, on the basis of the statistical significance, can be made that the tests are really slower, but the greater number of comparisons do go in one direction, and so may appear to have some weight apart from the significance of the individual differences considered separately. This evidence is offset by the fact that the exceptions are strongly centered in one strain; they are not distributed at random as though due to irregular causes. The frequency distributions of each rat in the group of 'completes'

TABLE 15

*Showing the significance of the difference in the speed (centimeters per second) of the tests and controls; sexes and strains together. The differences favor the controls, but are not significant*

PERIOD	AV. SPEED (CMS. PER SEC.)		DIFF.	P. E.	DIFF./P. E.
	Tests	Controls			
All training . . . . .	2.982	3.203	+0.215	±0.488	0.44
Retention . . . . .	3.694	3.995	+0.301	±0.145	2.07

upon which these comparisons are based are given in figure 13 for *training* and *retention*.

The averages of the speed for each day separately of *training* and *retention* have been studied. When the sexes are put together all but one day in strain A, and two days in strain L show the controls with higher speeds; but every day in strain C shows the tests with higher speeds. This same general difference between the strains holds when each sex is averaged alone. So it appears that the averages for *all training* and *retention* represent differences that hold day by day for each strain, and they are not due to irregular circumstances effective only on some particular day or days. The findings are so unquestionably negative that it has not seemed necessary to present the tables.

This study of speed was undertaken to see if there was any difference in the general nature of the motor activity of the tests and controls apart from their success on the maze. In

each strain the tests took more time and covered more distance in making their trials, yet in two strains the tests' rate of motion was slower than the controls, while in one it was faster. It must be concluded that, whatever difference there is between

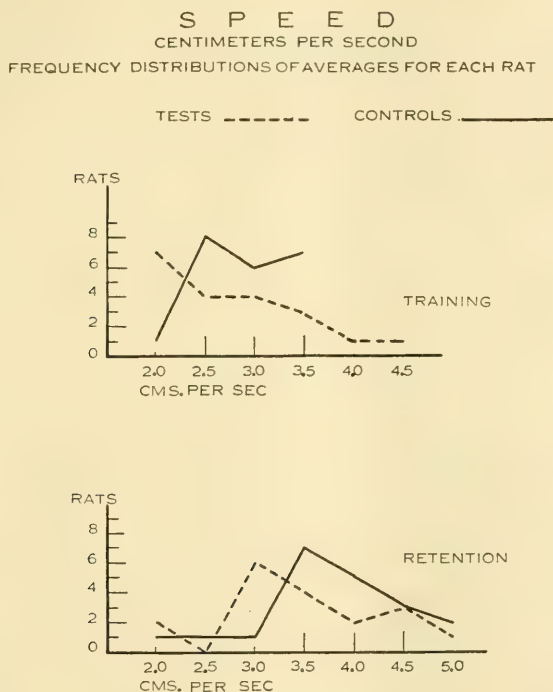


Fig. 13 Frequency distributions of the individual averages based on speed (centimeters per second). 'Completes' are used, males and females in all strains together. Units on the base lines are centimeters; the numbers given are the lower limits of the classes. Units on the vertical scale are the numbers of individuals. Broken lines show the tests; solid lines, controls. Based on distance as measured from the record sheets = about 1/13th actual speed.

the rats with normal and those with alcoholized grandparents, it is not a matter of the general rate of their motor activity, or of their hypo- vs. hyperkinetic nature.

5. *Comparison of the tests and controls on the basis of the numbers of errors*

a. *All types of errors together.* For the present purposes an error has been defined as a departure that extends more than half an inch from the true path as measured on the record sheets (approximately the length of a rat on the maze itself); it may involve passing a door it should enter, it may involve turning back in the true path. All types of errors, regardless of their length have been put together. Table 16 presents the error data summarized in the same way as the distance data in table 7; the numbers in the body of the table are averages of each rat's average per trial for the period and group indicated.

The table gives sixty-five 'plus' ratios and seven 'minus' ratios (six of which are due to the females in strain A); in sixty-five pairs of averages the tests have more errors than the controls, while in seven the controls have more errors than the tests. Table 17 gives the differences between the averages and the probable errors of the differences when all strains and the sexes are put together. *First half of training, omitting the first day, all training, and training and retention* give differences that are over three times their probable errors. For the *second half of training* the difference is only a little below three times its probable error (2.52 times). Figure 14 shows the distributions of the individual averages in the different groups of trials.

These results are in accord with all that has gone before and they follow those for distance so closely, quantitatively as well as qualitatively, that the comparisons based on each day by itself may be assumed to give the same results. Although these averages have been calculated, they are not presented; we can state that the supposition that they would give the same results is borne out by the averages themselves. It has not seemed necessary to even make the calculations of the averages of all rats in each group for each of the thirty-six trials by itself.

The variability of the tests, judged by the standard deviations of the average number of errors for each rat, is significantly greater than that of the controls in the *first half of training*

TABLE 16

Errors per trial for six groupings of trials; averages of each rat's average, based on 'completes' and 'incompletes.' A plus sign indicates that the average of the tests is higher than the average of the controls; a minus sign, that the average of the controls is higher. In most cases the tests made more errors than the controls

STRAIN	PERIOD	MALES			FEMALES			MALES AND FEMALES		
		Tests	Controls	Ratios	Tests	Controls	Ratios	Tests	Controls	Ratios
A	First half training . . . . .	5 rats 13.9	3 rats 7.1	(+)1.96	3 rats 7.6	7 rats 6.8	(+)1.12	8 rats 12.1	10 rats 6.9	(+)1.76
	Second half training . . . . .	4.4	2.7	(+)1.63	3.5	4.4	(-)1.26	4.1	3.9	(+)1.03
	Omitting first day . . . . .	7.0	3.8	(+)1.84	3.8	4.2	(-)1.10	5.8	4.1	(+)1.41
	All training . . . . .	9.2	4.9	(+)1.87	5.7	5.6	(+)1.02	8.2	5.4	(+)1.19
	Retention . . . . .	3.6	2.6	(+)1.38	2.0	3.2	(-)1.60	2.8	2.9	(-)1.04
C	Training and retention . . . . .	6.0	4.1	(+)1.46	4.4	4.7	(-)1.07	5.4	4.4	(+)1.21
	First half training . . . . .	4 rats 9.0	3 rats 5.6	(+)1.61	1 rat 13.8	2 rats 6.8		5 rats 10.0	5 rats 6.1	(+)1.64
	Second half training . . . . .	2.7	1.0	(+)2.70	3.8	0.6	(+)6.33	2.9	0.8	(+)3.47
	Omitting first day . . . . .	3.5	1.7	(+)2.05	5.7	2.2	(+)2.59	4.0	1.9	(+)2.07
	All training . . . . .	5.8	3.2	(+)1.81	8.8	3.7	(+)2.38	6.4	3.4	(+)1.88
L	Retention . . . . .	1.4	1.5	(-)1.07	2.7	1.1	(+)2.45	1.7	1.4	(+)1.26
	Training and retention . . . . .	4.7	2.7	(+)1.74	6.8	2.8	(+)2.43	5.2	2.7	(+)1.89
	Frist half training . . . . .	7 rats 9.8	4 rats 8.6	(+)1.14	5 rats 7.8	6 rats 6.4	(+)1.22	12 rats 8.9	10 rats 7.3	(+)1.22
	Second half training . . . . .	3.5	2.7	(+)1.29	2.5	2.1	(+)1.19	3.1	2.3	(+)1.30
	Omitting first day . . . . .	4.3	3.5	(+)1.22	3.3	2.9	(+)1.14	3.8	3.1	(+)1.24
All	All training . . . . .	6.7	5.7	(+)1.17	5.1	4.4	(+)1.16	6.0	4.9	(+)2.23
	Retention . . . . .	2.0	1.2	(+)1.67	2.3	1.3	(+)1.77	2.2	1.3	(+)1.70
	Training and retention . . . . .	5.4	3.9	(+)1.38	4.2	3.4	(+)1.23	4.6	3.4	(+)1.36
	First half training . . . . .	11.0	7.2	(+)1.51	8.5	6.6	(+)1.28	25 rats 10.1	25 rats 6.9	(+)1.47
	Second half training . . . . .	3.6	2.2	(+)1.62	2.9	3.0	(-)1.02	3.4	2.7	(+)1.25
All	Omitting first day . . . . .	5.0	3.0	(+)1.64	3.7	3.4	(+)1.09	4.5	3.3	(+)1.38
	All training . . . . .	7.3	4.7	(+)1.55	5.7	4.9	(+)1.17	6.8	4.8	(+)1.41
	Retention . . . . .	2.3	1.9	(+)1.25	2.3	1.9	(+)1.19	2.3	1.9	(+)1.22
	Training and retention . . . . .	5.3	3.5	(+)1.52	4.6	3.7	(+)1.23	5.0	3.6	(+)1.37



(the difference is 4.13 times its probable error) and *omitting the first day* (2.95 times the probable error). In the other groups of trials there is no significant difference (table 18).

TABLE 17

*Showing the significance of the differences between the averages of the number of errors made by the tests and controls in each of the six groupings of the trials. These differences are taken from the averages for all strains and both sexes in table 16. Plus signs indicate that the tests made more errors than the controls. There is a strong tendency for the tests to make more errors, and the differences in the averages are in most cases significant*

PERIOD	DIFF. P. E.	DIFF./P. E.
First half training . . . . .	+3.25±0.62	5.24
Second half training . . . . .	+0.68±0.27	2.52
Omitting first day . . . . .	+1.26±0.31	4.06
All training . . . . .	+1.97±0.43	4.58
Retention . . . . .	+0.41±0.27	1.51
Training and retention . . . . .	+1.35±0.27	5.00

TABLE 18

*Standard deviations of the averages of tests and controls based on the number of errors per trial in each of the six groupings of trials; calculated from the averages for each rat given in table C in the appendix. When the standard deviation of the tests is greater than that of the controls, the difference is called plus; when the controls have a greater standard deviation the difference is marked minus. The only significant differences are in the direction of greater variability in the average number of errors on the part of the tests*

PERIOD	TESTS	CONTROLS	DIFF. P. E.	DIFF./P. E.
First half training . . . . .	3.95	2.13	+1.82±0.44	4.13
Second half training . . . . .	1.35	1.60	-0.25±0.20	1.23
Omitting first day . . . . .	1.90	1.28	+0.62±0.21	2.95
All training . . . . .	2.29	2.19	+0.10±0.26	1.38
Retention . . . . .	1.21	1.26	-0.05±0.18	0.27
Training and retention . . . . .	1.38	1.09	+0.29±0.19	1.52

*b. Each type of error separately.* The different types of errors seem to provide an excellent opportunity for a more analytical study of the behavior of the test and control rats; it seemed possible that the differences in the time and distance records might be accompanied by differences in the kind as well as the

# ERRORS PER TRIAL

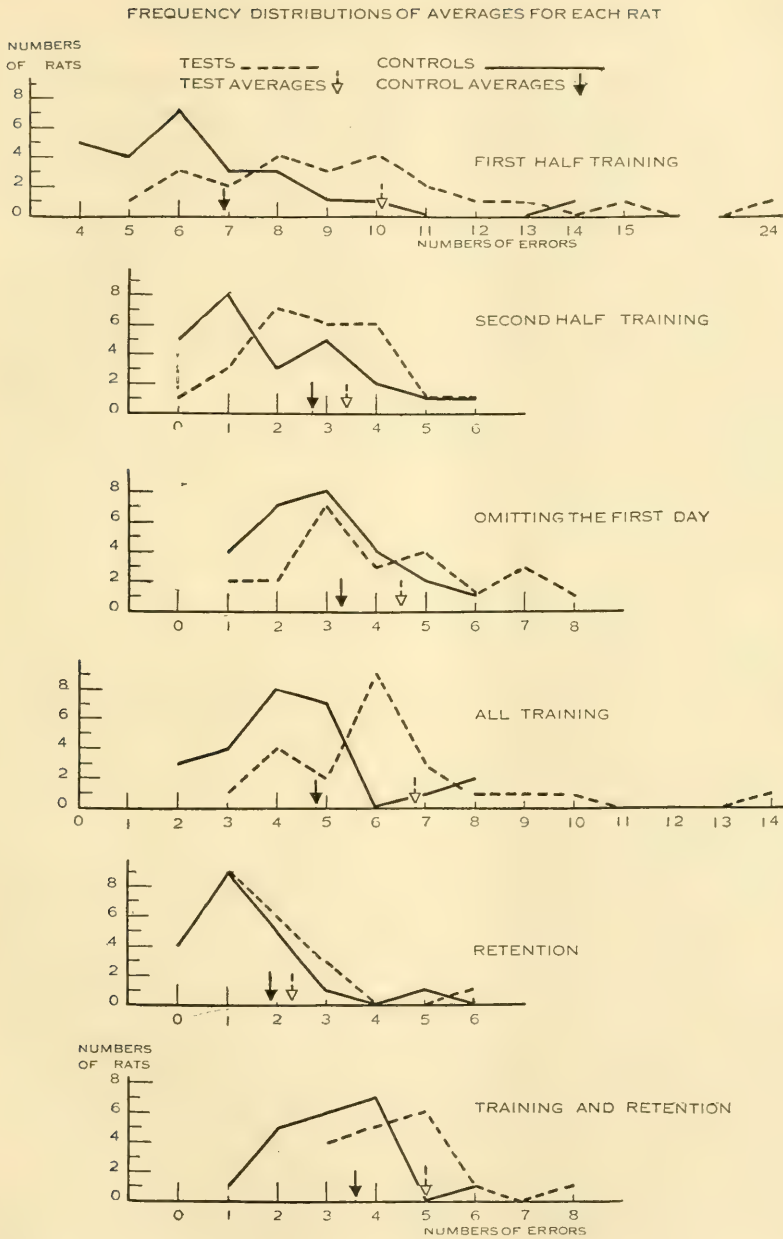


Fig. 14 Frequency distributions of the individual averages of the numbers of errors per trial, in the six groupings of the trials. Broken lines show the tests; solid lines, the controls. Units on the base line are numbers of errors; units on the vertical scale are numbers of rats. 'Completes' and 'incompletes' have been used, with the males and females in all strains together.

numbers of errors made. Six types of errors have been recognized: type 1, passing a door that should be entered; type 2, turning the wrong way upon entering a door; type 3a, turning back on the correct path; type 3b, turning back on the wrong path; type 4a, retracing the correct path through a door; type 4b, retracing the wrong path through a door. About 70 per cent of the errors made are of types 1 and 2. No clear difference between tests and controls was found on the basis of the percentage occurrence of each type of error in the total number of errors. When the strains and sexes are put together, for eighteen of the twenty-four trials in *training*, the averages of the tests for errors of type 1 are higher than the corresponding control averages; on twenty-one of the twenty-four trials the averages of the tests for errors of type 2 are higher than the corresponding averages of the controls. (These statements are made from curves based on the 'completes.') For errors of type 3a the tests have higher averages on ten trials; for errors of type 3b, the tests have higher averages on eleven trials; for errors of type 4a, the tests have higher averages on ten trials; for errors of type 4b, the tests have higher averages on seven trials (controls higher on one trial). The last three types of errors occur so infrequently that in most cases after the first seven trials there is no difference between the averages of the tests and controls. In the initial trial for each type the tests have more errors. If the comparisons in figure 14 had been based upon errors of types 1 and 2 alone, the differences would have been accentuated. The conclusion may be drawn that, although there does not seem to be much difference between tests and controls in the number of the more unusual errors, the tests are clearly less successful in eliminating the more persistent errors (types 1 and 2). This conclusion is strengthened by the following analysis.

*c. The number of trials before each type of error was eliminated.* For the purposes of this comparison, an error was said to be eliminated when it occurred only once in four successive trials; these four trials are included in the number of trials before the error was eliminated.

	TYPE OF ERROR					
	1	2	3a	3b	4a	4b
Tests . . . . .	12.8	15.5	10.3	5.3	5.1	4.7
Controls . . . . .	9.6	14.4	9.4	4.9	5.5	4.7
Difference . . . . .	+3.2	+1.1	+0.9	+0.4	-0.4	0.0

The controls eliminated errors of type 1, three trials sooner; they eliminated errors of types 2 and 3a about one trial sooner than the tests. The elimination of the other types of errors is very slightly different for the tests and controls. Comparing the numbers of test and control rats that did not eliminate each type of error in the twenty-four trials in *training*, the following numbers are found:

	TYPE OF ERROR					
	1	2	3a	3b	4a	4b
Number of rats failing to eliminate:						
Tests . . . . .	3	14	0	0	0	0
Controls . . . . .	0	10	1	0	0	0

In types 1 and 2, three and four more tests than controls failed to eliminate the respective types of errors. Although the controls eliminated errors of type 3a on the average sooner than the tests, one of the control rats did not eliminate this type of error at all. Otherwise, the numbers of rats that did eliminate the respective types of errors and the rapidity in doing so fully agree in favoring the controls.

The comparisons based on the error data may be summarized as follows: the tests made more errors; this difference was due mainly to errors of types 1 and 2, as approximately equal numbers of errors of the other two types were made by the tests and controls; the tests were slower in eliminating errors of types 1 and 2, but very little difference was found in the number of trials required for the elimination of errors of the other types. The variability in the number of errors is significantly greater for the tests for two groups of trials (*first half of training* and *omitting first day*).



TABLE 19

*Numbers of perfect trials; averages of the number made by each rat, based on 'completes' and 'incompletes.' The numbers of rats are given in parentheses. The averages are compared by their ratios; plus signs are given when the averages of the controls are higher than those of the tests, and minus signs when the averages of the tests are higher than the controls. When the sexes and strains are put together the difference for training is  $+1.28 \pm 0.40$ , which is 3.07 times its probable error; the difference for retention is  $+1.62 \pm 0.40$  which is 4.05 times its probable error. There is a significantly greater number of perfect trials made by the controls than by the tests*

STRAIN	PERIOD	MALES			FEMALES			MALES AND FEMALES		
		Tests	Controls	Ratios	Tests	Controls	Ratios	Tests	Controls	Ratios
A	Training Retention	1.20(5)	1.67(3)	(+).1.39	1.00(3)	0.85(7)	(-).1.17	1.12(8)	1.10(10)	(-).1.10
		1.33(3)	2.33(3)	(+).1.75	2.67(3)	2.00(4)	(-).1.33	2.00(6)	2.14(7)	(+).1.07
C	Training Retention	1.00(4)	6.67(3)	(+).6.67	1.00(1)	6.50(2)	(+).6.50	1.00(5)	6.60(5)	(+).6.60
		2.67(3)	5.33(3)	(+).1.99	3.00(1)	5.50(2)	(+).1.83	2.75(4)	5.40(5)	(+).1.96
L	Training Retention	2.14(7)	2.50(4)	(+).1.16	1.60(5)	2.50(6)	(+).1.56	1.91(12)	2.50(10)	(+).1.31
		3.00(4)	5.00(2)	(+).1.66	2.80(5)	5.33(6)	(+).1.90	2.89(9)	5.25(8)	(+).1.82
All	Training Retention	1.66(16)	3.50(10)	(+).1.84	1.33(9)	2.27(15)	(+).1.71	1.48(25)	2.76(25)	(+).1.80
		2.40(10)	4.12(8)	(+).1.71	2.78(9)	4.25(12)	(+).1.53	2.58(19)	4.20(20)	(+).1.63

### 6. Comparison of the tests and controls on the basis of perfect trials

*a. Number of perfect trials.* The error data provide another set of comparisons, namely, the trials on the 0 point of the scale of errors, those with no errors, or perfect trials. Table 19 presents the averages of the number of perfect trials for each rat for *training* and *retention*, with the strains separately and together. In this table the plus sign is given to the ratios when the tests' averages are *lower* than the controls'.

TABLE 20

*Standard deviations of the numbers of perfect trials when all strains and both sexes are put together; based on 'completes' and 'incompletes.' The differences and their significance in terms of their probable errors are given. The plus signs indicate that the tests are less variable than the controls. The tests are significantly less variable than the controls in the number of perfect trials they made in training; in retention the difference lies in the same direction and is very nearly great enough to be considered significant*

PERIOD	TESTS	CONTROLS	DIFF.	DIFF./P.E.
Training . . . . .	1.60	2.50	+0.90±0.30	3.00
Retention . . . . .	1.49	2.20	+0.71±0.28	2.53

The ratios indicate that the tests had fewer perfect trials than the controls; the females in strain A make the only exception. Combining the strains removes all minus signs; that is, the tests in all cases have fewer perfect trials. The differences between the averages, when the sexes and strains are combined, favor the controls; for training, the difference is  $+1.28 \pm 0.40$ , which is 3.07 times its probable error; for retention,  $+1.62 \pm 0.40$ , which is 4.05 times its probable error. These differences are fully significant. In figure 15 the distribution of the numbers of perfect trials of each rat are shown for *training* and *retention*.

Compared by the standard deviations of the numbers of perfect trials, the controls are more variable than the tests (table 20). The difference is 3 times the probable error for *training* and 2.53 times for *retention*.

This is the only criterion that gives a significantly greater variability for the controls. But obviously this has a different

meaning from the variability in the other data. In both time and perfect trials the variability is controlled by the high variates, since the low variates are limited by the physical possibilities of speed in time, and in perfect trials, by the absence of a

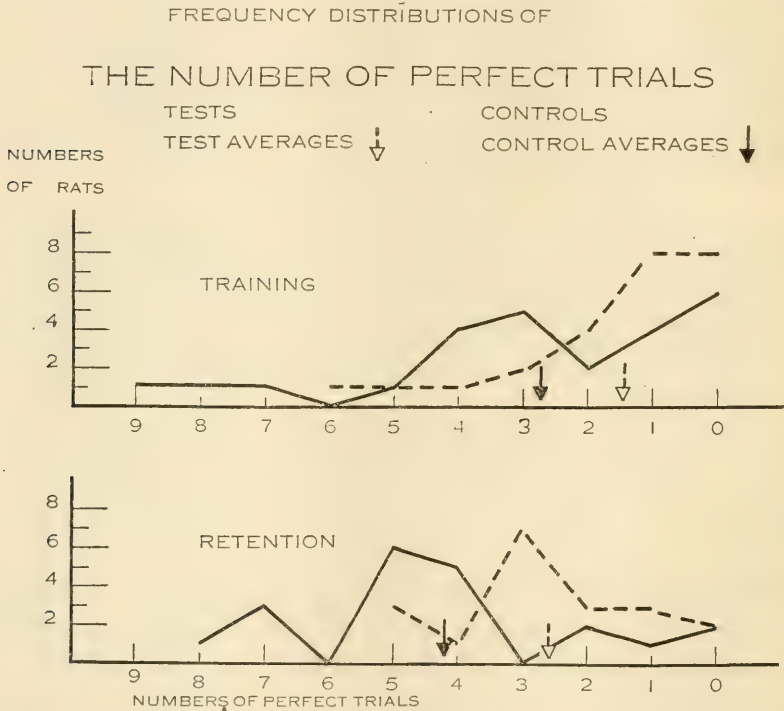


Fig. 15 Frequency distributions of the numbers of perfect trials made by each rat. Units on the vertical scale are numbers of rats, units on the base line are numbers of perfect trials. In order to keep inferiority at the right as in preceding charts, the scale reads from right to left. Broken line shows the tests; solid line, the controls. 'Completes' and 'incompletes' are used, with males and females in all strains together.

single perfect trial. A larger number of high variates, i.e., greater variability, is associated with inferiority in time; in perfect trials, with superiority.

*b. The number of trials before the first perfect trial.* The number of trials before the first perfect trial is a further basis for comparison of the tests and controls. Table 21 gives the average

number of trials before the first perfect trial for strains separately and together. The 'plus' before each ratio sign indicates that perfect trials appeared later in the training of the tests than in the training of the controls. In every case the averages of the tests are higher; that is, they took more trials before making their first perfect trial. When all the rats in each group are put together, the difference between the tests and controls is significant as tested by the probable errors ( $+5.66 \pm 1.66$ ; that is, 3.41 times the probable error). Two test rats and one control had no perfect trial during all the thirty-six trials. In calculating the mean number of trials before the first perfect trial these three rats were given the total number (thirty-six). Many more trials may have been required, however, before attaining a perfect trial. If these rats are excluded from the averages, the difference is still about the same ( $+5.09 \pm 1.44$ , which is 3.53 times the probable error). The frequency distribution of the numbers of trials, from which these averages have been calculated are given in figure 16. Besides having made fewer perfect trials, the tests required more trials before they made a perfect one.

c. *Time spent in running perfect trials.* A third criterion involving perfect trials is the time spent on running them. In figure 17 are shown the perfect trials classified according to the time spent; the frequencies are in terms of per cents of the total numbers of perfect trials. Thus the differences in the numbers of perfect trials for tests and controls is removed and the difference observed between the curves is due alone to the fact that the tests spent more time in running their perfect trials than did the controls. The curves are based upon 102 perfect trials for the tests and 163 for the controls; the average number of perfect trials per rat in *training* and *retention* together is, for the tests 4.08, for the controls 6.50. The average time spent on a perfect trial is, for the tests 8.48 seconds, for the controls 7.52 seconds; making a difference of  $0.96 \pm 0.25$ , which is 3.84 times its probable error and accordingly a significant difference.

The tests made fewer perfect trials, required more training before making the first one and took more time in running them than did the controls.



TABLE 21

*Averages of the number of trials before the first perfect trial, based on 'completes' and 'incompletes.' The averages are compared by their ratios; plus signs indicate that the test averages are higher; that is, the tests required more trials in order to make a perfect trial. The difference between the averages for all strains and both sexes is  $+5.66 \pm 1.66$ , which is 3.41 times the probable error. Two tests and one control did not make a perfect trial; in the averages these have been given the maximum of thirty-six days. If these are excluded from the averages that include both sexes and all strains, the averages become: tests 17.90, controls 12.81, with a ratio of  $(+1.397$  and a difference of  $+5.09 \pm 1.44$ , which is 3.53 times the probable error. The tests required more trials before making a perfect trial than did the controls*

STRAIN	MALES			FEMALES			MALES AND FEMALES		
	Tests		Ratios	Controls		Ratios	Tests		Ratios
	Tests	Controls		Tests	Controls		Tests	Controls	
A.....	29.25(4)	14.67(3)	(+1.38	24.00(3)	20.20(5)	(+1.19	21.85(7)	18.12(8)	(+1.20
C.....	17.25(4)	8.33(3)	(+2.07	23.00(1)	8.50(2)	(+2.70	18.40(5)	8.40(5)	(+2.19
L.....	16.33(6)	15.26(4)	(+1.07	21.00(5)	11.67(6)	(+1.80	18.45(11)	13.10(10)	(+1.41
All.....	17.71(14)	13.00(10)	(+1.36	22.22(9)	14.46(13)	(+1.51	19.48(23)	13.82(23)	(+1.41

FREQUENCY DISTRIBUTIONS OF  
THE NUMBER OF TRIALS  
BEFORE THE FIRST PERFECT TRIAL

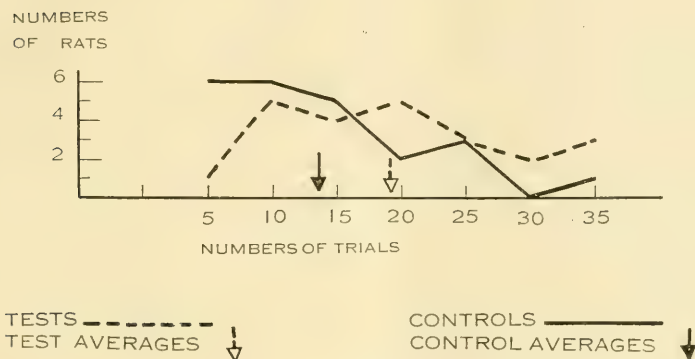


Fig. 16 Frequency distributions of the number of trials required before the first perfect trial. Units on the base line are trials; units on the vertical scale are individuals.

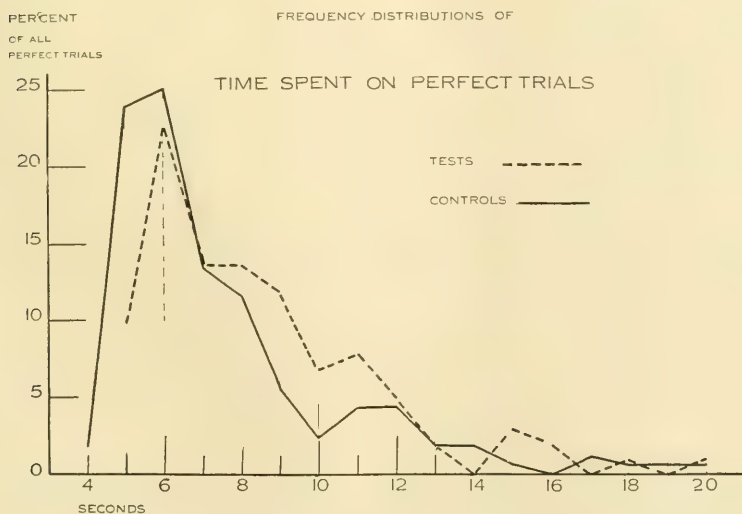


Fig. 17 Percentage distribution of the time spent in running perfect trials. Units on the base line are seconds, units on the vertical scale are per cent. The broken line shows the tests; the solid line, the controls.

## DISCUSSION

Although no general discussion of the literature upon experimental alcoholism is presented in this paper, reference must be made to one paper on account of the very close similarity of the present subject with some of the work therein described. Arlitt, '19 (pp. 41-44), has presented the results of training in a maze, the grandchildren of alcoholized white rats. The alcohol was administered with the food; doses of different sizes were given for different periods to various sets of animals obtained from a dealer. In order to eliminate strain differences, these sets were chosen at random; animals from the same source were used as controls. Three sets of rats are given in the third generation (the grandchildren of alcoholics by alcoholics) that are parallel to the ones we have described in this paper. Four of the nine grandchildren of rats that received 0.5 cc. of 90 per cent alcohol per day for five months did not succeed in making a single trip through the maze, so they are not included in the averages; the other five in this group were inferior to normals by each of the three criteria: total time spent, total number of errors made, and number of trials required to attain a certain degree of perfection in running the maze. The eleven grandchildren of rats that were given 0.25 cc. per day for thirty-nine days were inferior to normals in the time spent, and practically equal to normals in the number of errors and the number of trials required to master the maze. The eleven grandchildren of rats that were given 0.25 cc. per day for four months spent more time than the normal, but made fewer errors and learned the maze in fewer trials than the normals. These results are based on comparisons with five normal rats by means of group averages unchecked by probable errors of the averages or of the differences.

The irregularity in the results leads us to suspect two things: 1) that the differences between the averages do not have significance directly in proportion to their size, and that some of the differences observed would not be significant at all if compared with their probable errors; 2) that, although random selection of the rats to be treated and to be used as controls may have eliminated strain differences in the generation treated with

alcohol, the subsequent inbreeding has isolated strain differences which mask the effects of the alcohol in the third generation. The great mass of experiments upon artificial selection give strong support to the belief that strain differences are revealed at once when inbreeding is started from a general population; the great variety of characters that have yielded strain differences upon inbreeding leads to the opinion that they may be found even in the ability of white rats to learn a maze. Arlitt admits this point and used random selections of rats in the beginning, "In order to eliminate group or strain differences" (p. 6). The comparison of the second and third generations of her rats affords evidence that such genetic differences between the sets do exist: "The behavior of the third generation closely resembles that of their parents" (p. 41, second sentence). The sets in the third generation that were superior and inferior to normals came from parents that were respectively superior and inferior. If this doubt as to the elimination of strain differences is correct, even accepting all the differences in the averages as though statistically significant, the results cannot be considered significant. The normals are no longer truly controls, since there is involved in the third generation, a second source of difference whose effect cannot be separated from that of the alcohol. Although the final conclusion reached on this subject by Arlitt is in agreement with our own, namely, that alcoholization effects the learning ability of the grandchildren, we do not feel that this agreement really strengthens our evidence. It seems entirely possible that a repetition of Arlitt's experiments with the same procedure and methods throughout, and accordingly with the alcohol exerting the same effect, might give a different conclusion, due to the chance selection of a slow strain to be used as normals.

#### GENERAL SUMMARY

This paper deals with the maze-behavior of the second non-alcoholized generation after alcoholic treatment of both grandparents. The normal controls were the grandchildren of the non-alcoholized brothers and sisters of the rats that were given



the alcohol treatment. Brother-by-sister matings were made throughout. The rats were given three trials a day for eight days on a circular maze (*training*), and after thirty-one days (during which time they were trained in our multiple-choice apparatus) they were given four days more, twelve trials (*retention*). Sixty rats in this generation were trained, thirty-one tests and twenty-nine controls. The records of ten of these rats include one or more trials that were not successful; these rats have been called 'failure' rats. No satisfactory method of treating the successful records of these rats has been found; accordingly, they have been omitted from most of the summaries. In order to show that the results so obtained do not depend upon the omission of these rats, they have been included as well as excluded in the summaries based on the criterion of time.

1. During the period of rapid learning the test rats spent more time than the controls in running their trials. In reaching this conclusion, the averages of the tests have been compared with the averages of the controls in each of the following groupings of the data: males and females separately and together in each strain by itself and in all strains combined for each of the following groups of trials: *first half of training*, *second half of training*, *omitting the first day*, *training*, *retention*, *training and retention*, and each day of *training* and *retention* by itself. In the group of rats including all strains and both sexes the averages of the tests are higher in each of the eighteen groups of the trials. The probable errors of the differences between the averages of the tests and controls for the *first half of training*, *omitting the first day*, *training*, *training and retention*, first, second, third, fourth days of *training* and the fourth day of *retention*, show that these differences are significant. The frequency distribution of the ratios of all the test trials to corresponding points in an assumed standard learning curve differs from the frequency distribution of the ratios of all the control trials to the same standard learning curve. When these two distributions are compared by means of the  $\chi^2$  test, it is found that the probability

that they are not random samples of the same population is, at least, greater than 1,000,000 to 1.

2. In the first half of training the variability of the time averages is greater for the tests.

3. The test rats covered more distance than the controls, especially in the period during rapid learning. This is based on the same groupings of the sexes, strains, and trials as were used for the time data. When the strains and sexes are put together, the averages of the tests are higher than the controls for each of the eighteen groups of trials. The probable errors indicate that the differences are statistically significant for the following groups of trials: *first half of training, omitting the first day, training, training and retention*. The test of the similarity of the frequency distributions of the ratios of each trial of the tests and controls to the corresponding points in an assumed standard normal curve indicates that the chances against these two frequency distributions being random samples from the same population are, at least, more than 1,000,000 to 1.

4. There is no significant difference between the variability of the distance averages of the tests and controls for any of the different groupings of the trials.

5. The speed of running (number of centimeters per second) does not show any clear difference between the tests and controls. Although the tests ran slower than the controls in two strains, in the third the controls ran slower than the tests. This difference in the strains appeared consistently in all the different groupings of the trials, so the alcohol treatment of the grandparents does not seem to have any consistent influence upon the general rate of motor activity.

6. The time and distance data are closely correlated; this is equally true for the data from the tests and the controls; tests,  $r = 0.95$ ; controls,  $r = 0.96$ .

7. The test rats made more errors than the controls. This is shown by the averages based on the averages for the following periods which give full significant differences: *first half of training, omitting the first day, training, training and retention*. The variability of the tests is significantly greater than the controls

in the two groupings of the trials, the *first half of training and omitting the first day*. The averages for each day have not been calculated.

8. The difference between the tests and controls in the number of errors depends upon two of six types of errors. The tests make more errors of types 1 and 2, but there is no real difference in the numbers of errors of the other four types made by the tests and controls.

9. The tests required three more trials on the average to eliminate errors of type 1; the tests required one more trial to eliminate errors of type 2. The other types of errors were eliminated in about the same number of trials by each group. This is based on the averages of the number of trials before each rat eliminated each type of error; an error was said to be eliminated when it occurred only once in four successive trials.

10. Seven more tests than controls failed to eliminate errors of types 1 and 2; the only rat failing to eliminate the other types of errors was a control.

11. The tests made fewer perfect trials than the controls. This statement is based on the average numbers of perfect (errorless) trials in *training* and *retention* separately.

12. The tests required more trials before making the first perfect trial than did the controls.

13. The tests spent more time in running their perfect trials than did the controls; this is based on the average time spent on perfect trial by the two sets of rats.

We believe that the above points show that the test and control rats differ, as groups, in their behavior in the maze. From the standpoint of learning their way to the center and going there for food, the tests are less successful than the controls. The alcoholic treatment of the grandparents is the only basis upon which the rats have been divided into the two groups of tests and controls; therefore the alcoholic treatment appears to be responsible for the inferiority of the tests in running the maze. If this is true, a modification of the genetic basis of inheritance is demonstrated.

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## APPENDIX



TABLE A

*Averages of the time taken by each individual rat in each of the six groupings of the trials. The sign (F) indicates that this rat had one or more unsuccessful trials*

STRAIN		RAT NUM- BER	SEX	FIRST HALF OF TRAINING	SECOND HALF OF TRAIN- ING	OMIT- TING FIRST DAY	ALL TRAINING	RE- TENTION	TRAIN- ING AND RE- TENTION
A	Test (F)	1169	♂		28.61			12.23	
	Test	1170	♂	127.71	30.83	33.58	79.27	19.07	59.20
	Test	1192	♂	165.40	19.05	29.40	92.22	16.15	66.86
	Test	1193	♂	261.40	51.43	80.76	156.41	36.93	116.58
	Test	1351	♂	234.48	25.70	43.63	130.09		
	Test	1354	♂	300.18	34.01	100.10	167.10		
	Test	1168	♀	146.16	41.36	59.83	93.76	20.88	69.46
	Test	1277	♀	88.63	36.28	36.11	62.45	24.50	49.80
	Test	1279	♀		31.10	33.68		31.61	
	Test (F)	1352	♀	244.47	19.41	90.48	127.05		
	Test (F)	1353	♀	328.36	39.53	101.44	177.67		
	Control	1171	♂	128.26	12.27	28.06	70.26	22.66	54.39
	Control	1172	♂	105.65	23.66	36.34	64.65	10.80	46.70
	Control	1265	♂	114.88	19.05	22.94	69.05	45.95	61.35
	Control	1173	♀	136.53	36.63	45.95	86.58	37.05	70.07
	Control	1174	♀	110.71	18.35	31.80	64.53	23.70	50.92
	Control	1175	♀	100.93	24.93	28.08	62.93	17.55	47.80
	Control	1266	♀	87.51	45.33	37.41	66.42	18.21	50.35
	Control	1331	♀	203.28	72.65	56.45	137.96		
	Control	1332	♀	130.25	48.53	44.59	89.39		
	Control	1334	♀	71.08	37.21	32.36	54.15		
C	Test	1177	♂	141.10	14.45	21.08	77.77	13.15	56.23
	Test	1178	♂	136.83	20.23	24.53	78.53	13.76	56.94
	Test	1179	♂	106.02	20.13	34.56	63.07	10.64	45.59
	Test	1182	♂	192.45	9.03	15.99	100.74		
	Test	1183	♀	193.06	22.93	37.09	108.00	19.53	78.51
	Test (F)	1184	♀	173.63	16.68	37.03	95.15	13.66	67.98
	Control	1209	♂	75.68	10.73	12.95	43.20	21.58	35.99
	Control	1211	♂	109.18	10.00	14.58	59.59	65.70*	61.29
	Control	1213	♂	123.25	12.15	31.46	67.70	8.25	47.88
	Control	1208	♀	236.90	8.40	50.79	122.65	9.65	84.98
	Control	1210	♀	122.61	9.38	24.06	66.00	21.43	51.14
L	Tests	1311	♂		19.15			19.03	
	Tests	1313	♂	374.06	31.68	44.00	202.78	19.85	141.86
	Tests	1318	♂	333.08	23.60	35.25	178.34	35.43	130.70
	Tests	1255	♂	139.86	41.80	58.81	90.83	28.63	70.09
	Tests	1224	♂	251.68	39.73	56.95	145.71		
	Tests	1225	♂	191.71	55.43	71.75	123.57		
	Tests	1227	♂	329.08	35.95	52.59	182.52		

TABLE A—Continued

STRAIN		RAT NUM- BER	SEX	FIRST HALF OF TRAINING	SECOND HALF OF TRAIN- ING	OMIT- TING FIRST DAY	ALL TRAINING	RE- TENTION	TRAIN- ING AND RE- TENTION
L	Tests	1256	♀	143.36	36.63	48.86	90.00	15.18	65.06
	Tests	1257	♀	212.58	48.10	55.36	130.34	24.41	95.03
	Tests	1315	♀	413.43	22.93	85.68	218.18	31.18	155.84
	Tests	1316	♀	123.78	15.70	28.46	69.74	25.15	54.87
	Tests	1317	♀	173.71	11.45	24.38	92.58	20.00	68.38
	Tests (F)	1312	♀		17.91			24.68	
	Tests (F)	1314	♀	205.41	25.76	60.34	115.59	34.35	88.51
	Control	1306	♂	187.93	24.76	29.78	106.35	17.00	76.56
	Control	1310	♂	152.25	30.70	29.13	91.50	14.40	65.80
	Control	1194	♂	221.75	32.70	43.90	127.22		
	Control	1195	♂	122.31	15.50	20.18	68.91		
	Control (F)	1305	♂	175.18	36.38	65.36	102.76	16.55	73.20
	Control (F)	1247	♂	184.74	20.36	40.65	98.98	12.56	69.35
	Control (F)	1196	♂	207.76	32.52	51.59	120.14		
	Control	1244	♀	154.26	16.36	28.35	85.31	14.65	61.76
	Control	1703	♀	66.90	13.12	19.47	40.00	9.47	29.82
	Control	1304	♀	113.91	37.07	32.42	75.49	19.10	56.69
	Control	1307	♀	182.56	15.28	29.22	99.42	11.95	70.26
	Control	1308	♀	63.41	21.76	33.92	42.59	17.47	34.21
	Control	1309	♀	101.06	11.78	25.10	56.42	13.07	41.97
	Control (F)	1246	♀	467.28	15.40	131.81	241.34	35.41	172.69

\* Sick.

TABLE B

*Averages of the distance covered by each rat in each of the six groupings of the trials, and the speed in terms of centimeters per second. The sign (F) indicates that this rat belongs to the 'failure' group*

STRAIN		RAT NUMBER	SEX	DISTANCE PER TRIAL						SPEED (CENTI-METERS PER SECOND)	
				First half of training	Second half of training	Omitting first day	All training	Retention	Training and retention	Training	Retention
A	Test	1170	♂	169.8	99.0	99.3	134.4	63.4	110.7	3.298	3.486
	Test	1192	♂	207.5	87.2	109.1	147.4	78.9	124.5	3.738	4.973
	Test	1193	♂	291.2	165.2	189.7	228.2	135.1	197.1	2.528	3.572
	Test	1351	♂	302.0	103.0	124.3	202.5				
	Test	1354	♂	355.5	109.8	159.4	232.6				
	Test (F)	1169	♀								
	Test	1168	♀	226.1	116.5	136.9	171.3	77.0	139.8	2.410	3.754
	Test	1277	♀	121.7	71.9	75.6	96.8	59.0	84.2	2.099	2.714
	Test	1279	♀		79.3	81.0		72.5		2.517	2.398
	Test (F)	1352	♀								
	Test (F)	1353	♀								
	Control	1171	♂	189.7	63.8	95.5	126.7	86.0	113.1	3.978	4.005
	Control	1172	♂	152.9	96.8	112.3	124.9	51.8	100.5	3.159	5.017
	Control	1265	♂	136.0	70.5	76.9	103.2	88.5	98.3	3.511	2.398
	Control	1173	♀	204.3	131.3	132.6	167.8	121.9	152.5	2.928	3.260
	Control	1174	♀	146.7	84.0	101.3	115.4	78.3	103.0	3.401	3.656
	Control	1175	♀	163.0	101.5	100.4	132.2	75.3	113.2	3.510	4.452
	Control	1266	♀	150.5	89.0	80.2	119.7	58.1	99.1	2.498	3.722
	Control	1331	♀	205.5	138.8	122.0	171.9				
	Control	1332	♀	124.7	100.5	104.5	112.6				
	Control	1334	♀	127.5	106.6	98.4	117.1				
C	Test	1177	♂	187.2	73.1	85.1	130.2	64.5	108.3	4.149	5.041
	Test	1178	♂	199.0	101.5	99.4	150.3	61.3	120.4	3.928	4.687
	Test	1179	♂	167.3	78.0	103.2	122.6	50.4	98.5	3.202	4.792
	Test	1182	♂	160.6	55.2	123.3	107.9				
	Test	1183	♀	264.0	99.4	127.2	181.7	80.0	147.8	3.883	4.250
	Test (F)	1184	♀								
	Control	1209	♂	127.8	47.9	52.1	87.8	57.3	77.6	3.850	3.677
	Control	1211	♂	128.3	43.9	53.3	86.0	79.4	83.8	3.512	2.547
	Control	1213	♂	142.5	51.2	75.6	96.8	39.6	77.7	3.215	5.137
	Control	1208	♀	144.0	42.9	75.9	93.4	40.1	75.6	3.664	4.390
	Control	1210	♀	165.6	41.6	66.1	103.6	64.3	90.5	3.522	3.513

TABLE B—Continued

STRAIN		RAT NUMBER	SEX	DISTANCE PER TRIAL						SPEED (CENTI-METERS PER SECOND)	
				First half of training	Second half of training	Omitting first day	All training	Retention	Training and retention	Training	Retention
L	Test	1311	♂		58.8			54.1		2.784	3.082
	Test	1313	♂	205.0	81.5	92.9	143.2	61.0	115.8	2.600	3.451
	Test	1318	♂	246.6	72.6	84.7	159.6	77.4	132.2	2.454	2.451
	Test	1255	♂	197.0	115.2	127.4	156.1	50.3	120.8	2.360	3.682
	Test	1224	♂	202.8	111.5	109.8	157.1				
	Test	1225	♂	170.2	139.6	129.3	154.9				
	Test	1227	♀	240.5	101.1	195.2	170.8				
	Test	1256	♀	167.3	84.7	94.7	126.0	54.0	102.0	2.099	4.167
	Test	1257	♀	201.2	98.5	111.4	149.8	83.0	127.5	2.038	3.845
	Test	1315	♀	246.2	80.6	108.8	163.4	91.3	139.3	2.222	3.314
	Test	1316	♀	143.2	59.5	82.0	101.4	77.0	93.2	3.113	3.478
	Test	1317	♀	159.5	48.7	60.3	104.1	58.0	88.7	3.426	3.050
	Test (F)	1312	♀								
	Test (F)	1314	♀								
	Control	1306	♂	181.0	80.2	81.2	130.6	57.2	106.1	2.659	4.399
	Control	1310	♂	132.3	90.4	79.2	111.3	47.6	90.0	2.576	3.942
	Control	1194	♂	278.8	101.4	132.5	190.1				
	Control	1195	♂	144.2	54.1	68.9	99.2				
	Control (F)	1305	♂								
	Control (F)	1247	♂								
	Control (F)	1196	♂								
	Control	1244	♀	157.5	62.3	70.4	109.9	47.6	89.1	2.817	3.687
	Control	1303	♀	103.5	53.1	59.4	78.3	47.6	68.0	3.290	4.445
	Control	1304	♀	160.9	113.0	97.4	105.7	67.1	92.8	2.844	3.870
	Control	1307	♀	205.5	58.4	79.1	131.5	54.3	105.7	2.912	4.695
	Control	1308	♀	111.2	67.5	81.2	89.4	66.0	81.6	2.984	4.591
	Control	1309	♀	116.0	52.0	65.5	85.3	55.1	75.2	3.358	4.511
	Control (F)	1246	♀								



TABLE C

*Averages of the number of errors made by each rat in each of the six groupings of the trials*

STRAIN		RAT NUM- BER	SEX	FIRST HALF OF TRAIN- ING	SECOND HALF OF TRAIN- ING	OMIT- TING FIRST DAY	ALL TRAIN- ING	RETEN- TION	TRAIN- ING AND RETEN- TION
A	Test	1170	♂	6.7	3.4	8.1	5.1	1.7	4.0
	Test	1192	♂	10.1	3.3	4.8	6.7	2.7	5.4
	Test	1193	♂	12.7	6.9	7.9	9.8	6.4	8.7
	Test	1351	♂	24.8	4.0	7.6	14.4		
	Test	1354	♂	15.6	4.6	6.6	10.1		
	Test (F)	1169	♂						
	Test	1168	♀	10.0	4.6	5.2	7.3	2.7	5.7
	Test	1277	♀	5.2	2.9	3.2	4.1	1.4	3.2
	Test	1179	♀		2.9	3.0		2.0	
	Test (F)	1352	♀						
	Test (F)	1353	♀						
	Control	1171	♂	8.5	1.6	3.6	5.0	2.9	4.3
	Control	1172	♂	6.9	3.6	4.6	5.2	1.6	3.9
	Control	1265	♂	6.0	3.0	3.1	4.5	3.4	4.1
	Control	1173	♀	8.9	5.9	5.7	7.4	5.8	6.9
	Control	1174	♀	6.7	2.7	3.5	4.7	2.7	4.0
	Control	1175	♀	6.5	3.7	3.6	5.1	2.8	4.4
	Control	1266	♀	5.4	4.0	3.1	4.7	1.4	3.6
	Control	1331	♀	9.2	6.9	5.7	8.0		
	Control	1332	♀	4.7	3.7	4.2	4.2		
	Control	1334	♀	6.2	4.2	4.0	5.2		
C	Test	1177	♂	10.3	2.5	3.6	6.4	1.6	4.8
	Test	1178	♂	9.7	3.5	3.8	6.6	1.7	4.9
	Test	1179	♂	8.6	3.2	4.7	5.9	1.0	4.3
	Test	1182	♂	7.4	1.6	2.1	4.5		
	Test	1183	♀	13.8	3.8	5.7	8.8	2.7	6.8
	Test (F)	1184	♀						
	Control	1209	♂	4.8	0.9	1.3	2.9	1.4	2.4
	Control	1211	♂	4.8	0.7	1.2	2.7	2.8	2.8
	Control	1213	♂	7.1	1.4	2.7	4.1	0.4	2.9
	Control	1208	♀	6.1	0.7	2.3	3.4	0.6	2.4
	Control	1210	♀	7.5	0.5	2.1	4.0	1.7	3.2
L	Test	1311	♂		1.2			1.2	
	Test	1313	♂	9.5	2.6	3.6	6.0	1.6	4.5
	Test	1318	♂	11.4	2.4	3.0	6.9	2.2	5.7
	Test	1255	♂	9.6	4.8	5.5	7.2	3.2	5.9
	Test	1224	♂	8.6	4.6	1.8	6.6		
	Test	1225	♂	8.1	5.6	7.8	6.3		
	Test	1227	♂	11.8	3.3	4.2	7.6		

TABLE C—Continued

STRAIN		RAT NUM- BER	SEX	FIRST HALF OF TRAIN- ING	SECOND HALF OF TRAIN- ING	OMIT- TING FIRST DAY	ALL TRAIN- ING	RETEN- TION	TRAIN- ING AND RETEN- TION
L	Test	1256	♀	7.0	2.8	3.2	4.9	1.2	3.7
	Test	1257	♀	8.7	4.2	5.0	6.4	3.4	5.4
	Test	1315	♀	10.2	2.7	3.8	6.4	3.0	5.3
	Test	1316	♀	6.3	1.8	2.9	4.1	2.7	3.6
	Test	1317	♀	6.9	0.8	1.6	3.9	1.4	3.0
	Test (F)	1312	♀						
	Test (F)	1314	♀						
	Control	1306	♂	8.7	2.7	3.2	5.9	1.2	4.4
	Control	1310	♂	5.9	1.3	2.7	4.5	1.2	3.4
	Control	1194	♂	14.0	3.6	6.1	8.8		
	Control	1195	♂	5.7	1.5	1.9	3.6		
	Control (F)	1305	♂						
	Control (F)	1247	♂						
	Control (F)	1196	♂						
	Control	1244	♀	7.6	1.8	2.5	4.7	0.8	3.4
	Control	1303	♀	4.1	1.2	1.7	2.7	0.4	1.9
	Control	1304	♀	6.3	4.0	4.2	5.9	2.7	4.8
	Control	1307	♀	10.1	1.4	2.8	5.7	1.3	4.3
	Control	1308	♀	4.9	2.8	3.5	3.9	1.6	3.1
	Control	1309	♀	5.6	1.4	2.5	3.5	1.2	2.7

Resumen por el autor Arch. E. Cole,  
University of Wisconsin.

Sobre la adquisición del oxígeno por ciertos animales que viven  
en el agua desprovista de oxígeno disuelto.

Birge y Juday han demostrado que durante una parte del año el agua del fondo del lago Mendota está desprovista de oxígeno disuelto, a pesar de lo cual ciertos animales continúan viviendo, aún durante este periodo. El problema de la adquisición de oxígeno por estos habitantes (particularmente las larvas de *Chironomus tentans*) durante el periodo de estancamiento ha sido objeto de estudio por el autor, quien ha llegado a las siguientes conclusiones: (1) *Chironomus tentans* no es anaerobio durante esta época, como demuestra su reacción con el cianuro potásico; (2) En el cuerpo de las larvas de chironómidos y en el de ciertos otros animales que participan del mismo habitat, ha encontrado el autor un complejo enzimático peculiar, el cual indica la posibilidad de la producción de oxígeno dentro del cuerpo, aun cuando esto no ha sido definitivamente demostrado; (3) Los fragmentos de plantas recojidos en el fondo del lago producen un poderoso agente oxidante. Los experimentos llevados a cabo conducen a la conclusión de que este agente oxidante es oxígeno atómico. Si esto es cierto, el oxígeno podría ser fácilmente utilizado por los animales que viven en la región estancada.

Translation by José F. Nonidez  
Cornell Medical College, New York

# OXYGEN SUPPLY OF CERTAIN ANIMALS LIVING IN WATER CONTAINING NO DISSOLVED OXYGEN

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TWO FIGURES

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## INTRODUCTION

Biologists have always displayed keen interest in problems relating to the metabolic activities of organisms which live in oxygen-free environments. It has been long known that certain bacteria normally live without oxygen; in fact, some are even unable to exist in its presence. Pflüger ('75) and Bunge ('83) showed that many invertebrates and even some vertebrates were able to carry on metabolic activities for a very limited period of time in the absence of oxygen. In 1908 Juday reported that several animals, including a mollusc, annelids, and the larvae of several insects, annually passed periods of three to four months at the bottom of Lake Mendota in an environment in which oxygen could not be demonstrated.



Birge ('07), investigating the inland lakes of Wisconsin, discovered that in certain of the deeper lakes there was a time each year when the lower water was entirely devoid of oxygen. This stagnation period he found to be a regular phase in the 'respiration cycle' of the lake, and he showed that it was brought about largely by physical factors.

Lake Mendota, which Birge studied with the greatest care, may be taken as an example. In the fall of the year, when the temperature of the lake has become nearly uniform at all depths, winds blowing across the lake set up currents which thoroughly 'mix' the water and equalize the amounts of dissolved gases from top to bottom. When the ice covers the lake the mixing ceases and the water becomes more or less thermally stratified. Toward spring, especially in the deeper parts of the lake, the water becomes stagnant, the oxygen being slowly used up by animals and decaying organic material. An excess of carbon dioxide accumulates in the place of the oxygen (Birge and Juday, '11). In the spring, when the ice leaves the lake, the wind by circulating the water again equalizes the amounts of dissolved gases and makes the lake inhabitable at all depths for aquatic animals.

As the season advances, the water near the surface of the lake warms most rapidly and a stratification again takes place. The upper water, becoming warmer and hence lighter, floats upon the lower, colder water. The wind is unable to force the warmer surface water down into the colder strata below, and consequently a sort of superficial circulation of the upper stratum of water results. The lower water, cut off from contact with the atmosphere, becomes stagnated and loses its oxygen. Three regions are thus formed in late summer and early autumn; the hypolimnion, or stratum of stagnation and low temperature; the epilimnion, or stratum of circulation and higher temperature (Birge, '03), and between these, the narrow thermocline (Birge, '03), or mesolimnion, characterized by a rapid transition in temperature.

During the summer stagnation period, most of the animals in Lake Mendota migrate from the hypolimnion to the epilimnion. This was shown to be true for fish by Pearse and Achtenberg ('20),

who set nets in the hypolimnion and rarely caught anything. The migration is due to the decrease of dissolved oxygen and the increase of carbon dioxide. The same investigators also showed that fish could not live for any great length of time in the stagnated region. They caught fish in the epilimnion, placed them in wire cages and then lowered the cages below the thermocline. They found that the fish died in about two hours, presumably from suffocation. However, there are animals in Lake Mendota (Juday, '08) which do not migrate from the stagnant, deeper water. Some of these even lead a rather active life throughout the stagnation period, in the soft bottom mud and in the water above it.

With these facts in mind, such questions as the following are suggested: How do these animals live? Do they use free oxygen during the stagnation? If so, where do they get it? Do they store it up or manufacture it? If so, how?

The solution of the problems raised was suggested by Dr. A. S. Pearse, to whom I am indebted for valuable suggestions and aid throughout this investigation. I am also indebted to President E. A. Birge and Mr. C. Juday for advice and for the opportunity to use certain limnological apparatus. Helpful suggestions have also been received from Dr. M. F. Guyer, Dr. W. J. Meek, Dr. A. S. Loevenhart, Dr. W. S. Marshall, and Dr. S. Tashiro. To Dr. G. H. Bishop I am indebted for help in the construction of apparatus.

#### REVIEW OF LITERATURE RELATING TO THE PHYSIOLOGY OF ANIMALS IN AN OXYGEN-FREE ENVIRONMENT

As early as 1804 Spallanzani showed that 'infusorial animalculae' could thrive when their oxygen supply was reduced to the minimum, and that they continued to give off carbon dioxide even when oxygen was entirely cut off. But, being contemporaneous with Lavoisier's notable work on oxidation, Spallanzani's experiments received little notice and the discoveries of Pasteur ('61) came as a revelation to physiologists who had generally believed that molecular oxygen was absolutely necessary for the metabolism of all organisms.

The organisms which Pasteur discovered were able to live in the absence of oxygen (in a medium where reduced methylene blue showed no signs of reoxidation) are now well known, and their relationship to oxygen is generally admitted. Since that time much work has been done to determine the resistance of various animals to the lack of oxygen, and the metabolic mechanism which enables certain of them to live for varying periods in its absence.

Thus Pflüger ('75) found that frogs were able to live for a period of twenty-four hours in an atmosphere of pure nitrogen. He determined that their carbon-dioxide output varied very slightly from that during a similar period in air.

Bunge ('83, '90) pointed out that an intestinal parasite of the cat, *Ascaris mystax*, can live for days in an oxygen-free saline solution and continue to produce carbon dioxide. He affirms that even when oxygen was available, the worm was unable to utilize it.

Pütter ('06, '08), working with leeches, Lesser ('10), with earthworms, and Weinland ('06), with pupae of flies, each pointed out that the metabolic processes could go on for varying periods of time in the absence of oxygen.

Krogh ('16), in summing up the work relating to metabolism of animals in an anaerobic environment, appears to believe with other workers in the field that in an absence of oxygen an incomplete breakdown, anoxybiosis, of food-stuffs takes place, resulting in the production of toxic products. Some animals are able to excrete these toxic substances more readily than others. An accumulation of the products of anoxybiosis probably inhibits the catabolic reactions which causes eventual death.

Weinland ('01) concluded from experiments on *Ascaris* living in an oxygen-free environment that the carbohydrates were chiefly catabolized, being broken down into carbon dioxide and fatty acids.

Packard ('07) found that fish lived longer in water free from oxygen if they were previously injected with carbohydrates, such as mannose or glucose. He assumed that the simple sugars act as depolarizers in the process of protoplasmic respiration.



This conclusion agrees with Matthew's ('05) theory of respiration. Matthew's hypothesis is that protoplasm by virtue of some unknown substance is such a powerful reducing agent that it is able to split up protoplasmic water into hydrogen and oxygen; the atomic oxygen going to oxidize substances, and the hydrogen, either combining with atmospheric oxygen, as in aerobic respiration or in anaerobic respiration, being set free as nascent hydrogen and combined with other substances. Atmospheric oxygen, according to this theory, acts merely as a depolarizer for the nascent hydrogen and the only difference between aerobic and anaerobic respiration is the manner in which the hydrogen is taken care of.

Packard ('07) believes that the carbohydrates injected into the fish take the place of atmospheric (molecular) oxygen, as the depolarizing agent for the removal of hydrogen.

Snyder ('12), arguing from this standpoint, attempts to prove that the anaerobic respiration was the primitive, fundamental type, and that the 'oxygen habit' was taken on during evolutionary development. Pütter ('05) also believes that anoxybiotic metabolism was the primitive type.

Juday ('08) found that many Protozoa, including members of the genera *Pelomyxa*, *Diffugia*, *Colpidium*, *Gyrocorys*, *Peranema*, *Coleps*, *Paramecium*, *Prorodon*, *Lacrymaria*, *Uronema* and *Monas*, lived normally for as long as four months in the lower waters of Lake Mendota in the absence of free oxygen. Living under these same conditions were other invertebrates, including the annelid worms *Tubiex* and *Limnodrilus*; a gastrotrich, *Chaetonotus*; an ostracod of the genus *Candona*; a small mollusc, *Pisidium idahoense*; the larvae of several chironomids, and occasionally the larvae of the black-winged orl fly, *Sialis infumata*. Juday kept several of these forms in the laboratory under oxygen-free conditions and found them to be practically unaffected by the absence of oxygen.

Maill and Hammond ('00) recognized the resistance of chironomid larvae to lack of oxygen. They put six larvae in bottles containing boiled water, superimposing a layer of carbonic acid, and sealing with a rubber stopper. Four of the six larvae



lived for forty-eight hours and one for nearly five days. Two pupated during the experiment. Pause ('18) saw an error in Maill and Hammond's work, in the fact that the boiled water might have contained small amounts of oxygen, so he constructed an apparatus similar to figure 1, page 302, in which he was able to determine the amount of oxygen dissolved in the water before and after each experiment. He kept the larvae of *Chironomus gregarius* in oxygen-free water for fifty-four hours. He found that the period of time during which the larvae were able to survive in the absence of oxygen varied directly with the amount of haemoglobin contained in the blood—the younger the larvae, the less haemoglobin and the more dependence upon free oxygen. He concluded, however, with Maill and Hammond ('00) that chironomid larvae were probably able to live without free oxygen by virtue of the fact that the haemoglobin could store up a sufficient supply to tide them over until oxygen was again available. This point will be discussed later.

It is a well-demonstrated fact that certain animals carry on an active existence at the bottom of Lake Mendota for considerable periods of time, living in water without detectable oxygen. There is no satisfactory explanation as to how this may be accomplished. This paper attempts to explain the possible sources of oxygen supply for these forms.

PHYSIOLOGY OF THE LARVA OF *CHIRONOMUS TENTANS* IN  
OXYGEN-FREE ENVIRONMENTS IN LAKE MENDOTA, AND  
THEORIES RELATING TO THE SOURCE OF ITS ENERGY

The greater part of the experimental work presented in this paper was done on the larvae of *Chironomus tentans* Fabricius, chiefly because of their size and abundance. Of the many species of the genus *Chironomus* occurring in Lake Mendota, the larvae of *C. tentans* are relatively abundant in the mud of the deeper parts of the lake, an average of over a hundred full-grown larvae being found per square meter at a depth of eighteen meters. For the methods used in obtaining larvae see Appendix, page 318.

The full-grown larvae are about 20 to 25 mm. in length and 3 mm. in diameter. They live in the mud at the bottom of the

lake, constructing tubes for themselves from small particles of organic debris and mud, the whole being cemented together by a secretion from their spinning glands. They remain in these tubes the greater part of the time, coming out occasionally to crawl over the surface of the mud for short distances, but soon burrow again. This fact was determined by observation of specimens kept in the laboratory under conditions as near normal as possible (Appendix, p. 318). The larvae of *C. tentans* are scavengers, their food consisting of mud containing small particles of organic material.

Chironomid larvae normally respire through blood-gills located on the last two segments of the body. The posterior part of the body keeps up rhythmical undulating movements which insure a continual fresh supply of water. Such movements continue when little, or even when no oxygen is present. Maill and Hammond ('00) state that the larvae of *Chironomus dorsalis* and *Chironomus plumosus*, large species which they studied, came to the surface when the oxygen supply was low to bathe their bodies in well aerated water. Such behavior has never been observed by the writer in connection with the larvae of *C. tentans*, either when the larvae were confined in oxygen-free water or kept in open jars in the laboratory. Furthermore, the limited swimming ability of this larva makes it highly improbable that those living at any considerable depth in the lake would be able to come to the surface or even travel very far in that direction.

The gills of the larvae of *Chironomus tentans*, which are merely outpocketings of the body wall, contain sinuses of the circulatory system and are continually filled with an ever-changing supply of blood. Oxygen, therefore, can be easily transferred from the water through the delicate gill membrane to the blood within.

The blood of the larvae of most species of the genus *Chironomus* contains haemoglobin, and therefore has a characteristically red color. Rollett ('61) was the first to show that the red pigment was haemoglobin. He obtained haemoglobin crystals and also showed that the blood was dichloric—that it gave a red color when light passed through a thick stratum, but

green if the stratum was very thin. Shortly after this, Lankester ('67) obtained the characteristic absorption spectrum of haemoglobin from chironomid blood. The larvae of *C. tentans* is richly supplied with this pigment, giving a bright scarlet color to the animal. The blood, then, by virtue of its contained haemoglobin has exceptional capacity for taking up oxygen dissolved in the surrounding water and for transporting it throughout the body. But during the stagnation period in Lake Mendota the supply of oxygen cannot be the same as during the preceding period when there is an abundance of dissolved oxygen. The oxygen has been all used up and an excess of carbon dioxide has taken its place.

This is well pointed out in the tables given by Birge and Juday ('11) for the year 1906. On April 20th the oxygen content of the water in Lake Mendota at a depth of 22 meters was 7.5 cc. per liter, and 8 cc. per liter at the surface. June 11th showed 1.8 cc. per liter at 22 meters in contrast to 6.3 cc. per liter at the surface. On July 10th there was no detectable oxygen at 22 meters, a trace at 20 meters, 0.1 cc. per liter at 18 meters and 7 cc. per liter at the surface. As the oxygen was progressively used up at higher levels, the oxygen-free stratum increased in thickness until, on September 30th, no oxygen was detected between a depth of 15 meters and the bottom. Following this time the upper surface of the oxygen-free belt dropped very slowly, due mainly to the decrease in temperature of the surface water, which allowed the heavy fall winds to circulate the water to greater depths. After the fall overturn, which came between October 8th and October 11th, there was 5.5 cc. of oxygen per liter at 22 meters. Between July 10th and October 8th no oxygen could be detected in the water at a depth of 22 meters, and for a somewhat shorter period, August 24th to October 8th, there was none at the 18-meter level. The work of Birge and Juday, extending through a period of over a dozen years, leaves no doubt that this is of annual occurrence. Titrations to determine the oxygen content of the deeper water of the lake were also made by the writer from time to time while these experiments were being carried on.

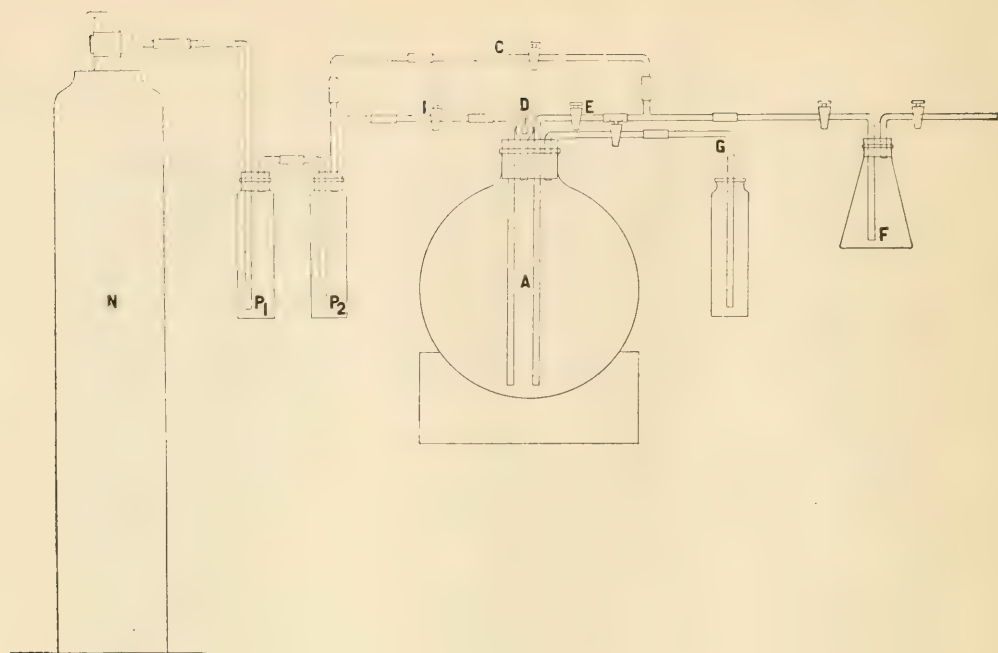


Chironomid larvae<sup>1</sup> live and are active in Lake Mendota in the mud at depths where there is no free oxygen throughout the summer stagnation period. This has been shown in two different ways. 1) When water and mud were pumped up from the stagnated region into bottles without contact with the air, several times chironomid larvae were also pumped up. (Appendix, p. 317). These bottles were tightly stoppered and set aside for observation. Control bottles without larvae were tested for the presence of oxygen, and none was found. For methods employed, see Appendix, p. 317. Observations were continued daily, and it was found that the larvae were continually active and lived for varying periods of time—one for fifty days—death presumably being due to the accumulation of waste products in the necessarily small container in which they were confined. 2) Chironomid larvae obtained from the stagnated region were confined in water which had been made artificially oxygen-free by prolonged boiling in the apparatus shown in figure 1, and described on page 318. These were active throughout their life in the flask—a period varying from one to three weeks.

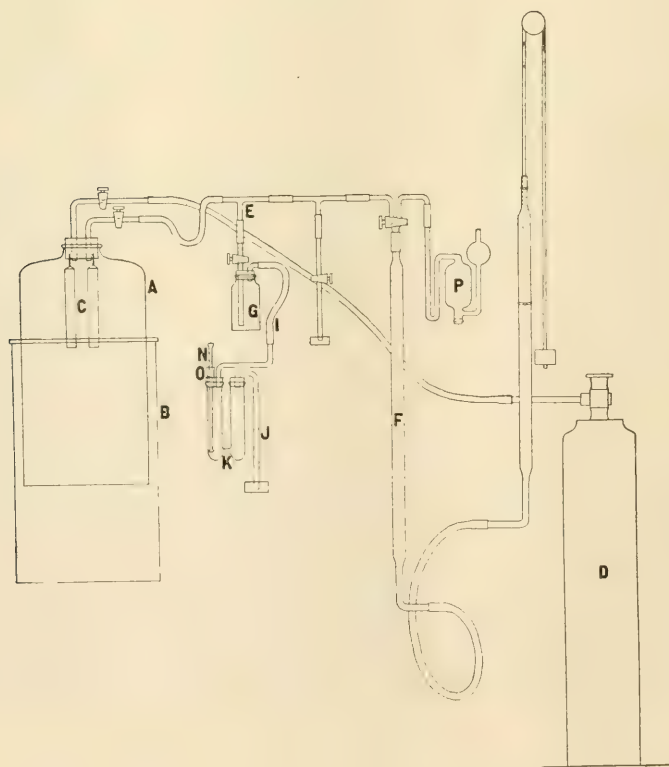
The difference of longevity of the larvae in 'natural' and 'artificial' oxygen-free water is probably due to the fact that the normal chemical content of the lake water and mud (which was put in every bottle to serve as food for the larvae) was disturbed in the process of boiling. Boiling certainly altered the character of the available food. The accumulation of waste products in each case was probably the immediate cause of death, as on opening the bottles the odors given off were exceedingly foul and smelled strongly of hydrogen sulphide. Additional support for this view comes from the fact that chironomid larvae used as controls in connection with the experiment just described were placed in bottles of well-oxygenated surface water, and it was found that they did not live appreciably longer and in some cases not as long as the larvae confined in oxygen-free water.

<sup>1</sup> Unless otherwise stated, 'chironomid larvae' when relating to the writer's experiments always refers to *Chironomus tentans* Fabr.





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If, then, chironomid larvae are active during the period when oxygen is absent, whence comes the oxygen which would liberate the energy stored up in their bodies? As was stated before, their activity does not consist of a few movements. They burrow through the mud, making new tubes, and even when lying in their tubes keep up an almost constant waving of the posterior parts of their bodies.

### *1. Anoxybiotic source of energy*

It is possible that the stagnated region at the bottom of Lake Mendota is an absolutely anaerobic environment, and that a fermentative process, an incomplete breakdown of the organic food-stuffs goes on within the body of a chironomid larva, which without oxygen would result in the production of energy. Such an explanation for energy production has already been cited (p. 296) relating to experiments on *Ascaris*, pupae of flies, etc. In the cases cited the energy needed is obviously small. It seems rather improbable that such a process could supply sufficient energy to meet the requirements of so active an organism as the chironomid larva under consideration and keep up the supply through so long a period of time. In the case of the chironomid larva, however, the writer found that individuals placed in oxygen-free water taken from the stagnant region of Lake Mendota succumbed to 0.000032 mol. KCN on the third day. Larvae exposed to weaker solutions of KCN lived longer, the length of time being roughly proportional to the strength of the KCN solution used. Inasmuch as KCN owes its poisonous properties primarily to its interference with oxidation processes and destruction of the respiratory center, the fact that the larvae were killed by the small amount of the cyanide indicates that an oxidation process was going on in their bodies even though oxygen could not be demonstrated in the surrounding medium.

Furthermore, if a process similar to the one that Weinland ('06) postulates for the pupae of flies is furnishing energy for the chironomid larva when its usual supply of oxygen is cut off, what explanation is to be made for the presence of haemoglobin? It does not seem reasonable that so complicated a mechanism

for transporting oxygen would be developed in an animal which would not use it all the time. Such a development is especially questionable when it is considered, as will be pointed out later, that the haemoglobin becomes functional only when the oxygen pressure is low.

The point made here is this; the incomplete breakdown of food-stuffs in the absence of oxygen within the body of the chironomid larvae offers a possible source of energy, though it is not thought to be the probable one.

## *2. Haemoglobin as a storehouse for oxygen*

It has been suggested by Maill and Hammond ('00) and suggested again by Pause ('18) that the haemoglobin of the blood may perhaps act as a storehouse for oxygen; that in times of sufficient oxygen supply the haemoglobin stores up oxygen which is then used when the supply in the surrounding medium has become exhausted.

Leitch ('16) estimated by the use of Krogh's microrespiratory apparatus that, "ten chironomus larvae weighing .16 gr. used .428 c. mgr. of oxygen in one minute. Chironomus has 50% of blood of an oxygen capacity of 6 c.c. pc., i.e. these ten larvae had .08 c.c. of blood whose total oxygen capacity was .0048 c.c. oxygen." This would suffice to store oxygen sufficient for about twelve minutes.

The ability of the haemoglobin of the chironomid larva to store up sufficient oxygen to last throughout the stagnation period is out of the question. However, the importance of haemoglobin must be admitted. In invertebrates, haemoglobin is found in the representatives of many species in many phyla, and its presence is always connected with a poorly oxygenated medium in which the various animals live. Undoubtedly, there is some correlation between the two facts. Leitch showed that down to an oxygen pressure of 7 mm. the chironomid larvae do not use their haemoglobin at all, the needs being supplied by physical solution. But below this point the haemoglobin is used to chemically combine with oxygen and transport it at oxygen pressures so low that the required amount is not supplied by simple physical solution.

### *3. Production of oxygen by enzymatic action*

It is well known that plants, by virtue of their chlorophyll and the sunlight, can combine inorganic compounds, building up complex organic compounds with the liberation of oxygen. This is considered to be due to an enzymatic substance within the plant. The oxygen, liberated in the atomic or active form, will as such oxidize alcoholic tincture of guaiacum to a compound having a blue color. It was therefore thought advisable to look into the enzyme content of the chironomid larvae, the object being to ascertain whether there might be enzymes in any way connected with their oxygen supply.

Most animal tissues contain an oxidizing enzyme which will split off atomic oxygen from hydrogen peroxide or an organic peroxide, and can be demonstrated by the oxidation of an indicator, such as guaiacum or benzidine, to a colored compound. On examination it was found that the chironomid larvae contained a substance which would do this. The blood from a chironomid larvae or an aqueous extract of their tissues would immediately oxidize alcoholic tincture of guaiacum on the addition of hydrogen peroxide or the organic peroxide found in old turpentine.

Whether or not this substance can be called an enzyme is largely a matter of definition. If the definition of an enzyme includes the statement that enzymes are destroyed by boiling, then this substance cannot rightly be considered as such. It was found that when the extract of a chironomid larva was raised to the boiling point and even kept at that temperature for some time, it still was capable of splitting off the oxygen from a peroxide. In this respect it is different from true enzymes. Iron seems to play an important rôle in the non-thermolabile peroxidase of the chironomid larvae. Iron is of course found in the haemoglobin of the chironomid blood, and was demonstrated qualitatively by the writer in the ash of the tissues. The exact rôle of iron in an enzyme is not known. Perrin ('05) and Bayliss ('14) agree that an iron-containing peroxidase consists of the union of an unstable colloidal ferric hydroxide with a stable colloid.



But obviously a peroxidase or any other catalyzing agent is of no use as an oxidizing agent without the pressure of a peroxide from which the oxygen may be split off. There are no peroxides in the blood of the chironomid larvae, otherwise guaiacum would be oxidized upon its addition. Neither were peroxides found in the extract of the soft tissues of the body.

However, the chironomid larvae contains a true enzyme whose reactions are quite different from the non-thermolabile enzyme just discussed. During the experiments on the larvae there were at times found to be small particles in the aqueous extract of the tissues which gave the blue color with tincture of guaiacum before the hydrogen peroxide was added. This matter was further investigated, and it was found by careful dissection and testing of various parts of the larvae that the reaction was only given by the chitin. Only the inner surface of the chitin was involved and the action was more pronounced when the chitin was thoroughly bruised or macerated. The separation of the chitin from the body tissues was accomplished by slitting the larvae longitudinally and scraping the organs out. This treatment removed the hypodermal cells which secreted the chitin. Then by grinding the chitin slightly in water with a pestle and adding the guaiacum, the blue color gradually appeared. This color is at first confined only to the inner surface of the chitin, but after a time gradually diffuses into the surrounding medium.

Tests showed that this reaction in the chitin is dependent upon an enzyme which is killed at a temperature of about 56°C. It was also found that if the extract was allowed to stand for some time before the guaiacum was added, the reaction was weakened. Standing for a period of twenty minutes greatly decreased the intensity, and if the extract remained for thirty minutes before the guaiacum was added, the reaction was barely if at all perceptible. The presence of such an enzyme in animals is quite unique. Generally a peroxide must be added to an animal extract before it will oxidize guaiacum.

In plant tissues such enzymatic reactions are very common. In apples, for example, a cut surface immediately oxidizes guaiacum, or if left by itself will turn brown, due to the oxidation of a chromogen compound which is brown when oxidized.

Such reactions in plants are explained by the presence of an enzyme which builds up a peroxide from a contained compound and the atmospheric oxygen. This peroxide, then, has its oxygen removed by a peroxidase, in the atomic or active form, and the atomic oxygen then oxidizes the color-producing compound. It is possible that an enzyme complex similar to that of plants exists in the chitin of the chironomid larvae.

That two enzymes, or an enzyme and an enzyme-like substance, are being dealt with is certain. For if a portion of the bruised chitin be treated with guaiacum, a blue color results. If another portion is raised to a temperature which is above the killing point of the enzyme, namely,  $56^{\circ}$ , and again a part tested with guaiacum, no color results. But if to the remaining heated portion, guaiacum and hydrogen peroxide are added, the guaiacum is at once oxidized to the blue color compound. These results indicate that a true thermolabile enzyme builds up a peroxide from which atomic oxygen is being split off by the action of an enzyme-like substance which is not killed by boiling.

A peculiarity exists here, however, in the fact that alcoholic tincture of guaiacum is the only one of the indicators tried which was oxidized by the reaction of this enzyme complex. No reaction was obtained with benzidine, *a*-naphthol, or *p*-phenylenediamine hydrochloride. It would be expected that active oxygen would affect oxidation indiscriminately. Bertrand ('96) showed that laccase acted upon hydroquinone and pyrogallol with ease, but not upon resorcinol or phloroglucinol. Bayliss ('14) seems to believe that there is a specificity here which "lies in the peroxide component of the oxidase system. If this be so it is possible that there is some intimate relationship necessary between the structure of the peroxide and the substrate in order that close connection may be possible, so that the active oxygen may enter into the immediate union with the latter." Such a close connection between the elements of the reaction may exist in the case of the enzyme complex in the chironomid larvae, which would account for the specificity shown.

It would seem that there might be some relation between the peculiar enzyme-complex found in the chironomid larvae living

at the bottom of Lake Mendota and the fact that for a portion of each year the water in which the larvae live contains no dissolved oxygen. If in the apple there is present an enzyme which can build up a peroxide by obtaining extra atoms of oxygen from the molecular oxygen in the air, may not the enzyme complex in the chironomid larvae have the ability to build up a peroxide by obtaining its extra atom of oxygen from some other substance contained in the water or mud? If such a peroxide were thus formed, the oxygen would be split from it by the peroxidase-like substance in the blood, and so be carried by the haemoglobin to the tissues where it could be utilized in physiological oxidation. Should this be the case, we have here, then, a system which would be slowly yet surely releasing oxygen for the oxidative processes which go on within the organism at the time when the supply of oxygen from the surrounding water fails. If this reaction can be shown to proceed when there is no oxygen in the surrounding medium, then we have an explanation for the source of oxygen used by the chironomid larvae during the stagnation period.

Experiments were made to test this hypothesis. (For apparatus and description of the experiment, see Appendix, p. 319.) It was found, however, when the molecular oxygen had been, removed both from the liquids involved and the atmosphere above them, that the enzyme complex was not capable of oxidizing the guaiacum. This, of course, would indicate that molecular oxygen was necessary in order to build up the peroxide component of the complex, as is true in the case of the enzyme complex found in the apple; and inasmuch as oxygen is not present during the stagnation period at the bottom of the lake where the chironomid larvae live, such a mechanism would mean nothing.

However, there are some points which must be taken into consideration here. Perhaps the concentration of carbon dioxide or nitrogen which was used to take the place of the oxygen-bearing atmosphere had an inhibiting effect on the action of the enzyme, or perhaps certain constituents were lacking which were necessary for the functioning of the enzymes; in other words that the exact substrate for the enzyme was not present.



But it seems very probable to the writer that some relationship exists between the two peculiar conditions—the presence of the enzyme complex in the chironomid larvae and the absence of the usual oxygen supply for a portion of each year. This seems doubly probable because of the fact that the ability of the chitin to oxidize guaiacum was found to be more intense in its action during those times of the year when dissolved oxygen was present in the lower water and gradually became weaker during the continuance of the stagnation period. It would seem that something was being stored when oxygen was abundant which was gradually used up when oxygen was not available. This material stored up cannot be in the form of a peroxide, because in the first place peroxides are toxic to living protoplasm, and, secondly, they would be broken down in the presence of the peroxidase-like substance found in the larvae's body. And if the stored substance is the enzyme itself, what avail would it be in the production of a peroxide, when it is dependent in its action on molecular oxygen which is absent during the stagnation period? A further investigation is already in progress by which the writer hopes to clear up this phase of the problem.

It has been suggested that the oxidation of guaiacum is not a sufficient index to the production of atomic oxygen, that it is merely an indication of the presence of a powerful oxidizing agent, which is similar in its action to atomic oxygen. The exact steps in the chemical reactions concerned in such an oxidation are not well understood. It has been argued that a peroxide may unite with the oxidizable substance, eliminating water in the reaction. In such a case the substance would be finally oxidized, but atomic oxygen would not necessarily be formed. However, neither hydrogen peroxide nor the organic peroxide found in old turpentine have any immediate effect on guaiacum, but this does not prove that some other highly complex organic peroxide might not have such an effect. In any event, the experiments performed by the writer show that the chironomid larvae contain, or are capable of forming in the presence of oxygen, an oxidizing substance capable of oxidizing guaiacum which would probably also be capable of oxidizing substances



in its body. Whether the production of an oxidizing agent is absolutely dependent upon the presence of molecular oxygen remains to be proven.

#### *4. Liberation of oxygen from decomposing plants*

It was noticed during the experiments just described that when oxygen was removed from the experimental chamber, although none of the chironomid tissue or chitin was able to oxidize the guaiacum, some of the plant debris in the mud did so. Such an oxidation would be a normal reaction in living chlorophyll-bearing plants, but for dead and partially broken-down plant fragments, it was considered to be rather unusual. It was thought that perhaps a few of the cells still contained functional chloroplasts which were carrying on photosynthesis in the presence of light, and so were giving off oxygen in the atomic form which was oxidizing the guaiacum.

To ascertain whether this was the case, a series of experiments were carried out similar to those cited on page 308 (also Appendix, p. 320). The experiments differed from those preceding, in that mud, containing decaying plant tissues, was used instead of the extract of chironomid larvae, and the experiments were carried out in darkness, the object being to find out if the plant debris was capable of oxidizing guaiacum in darkness as well as in the absence of molecular oxygen. One of a series of experiments was allowed to continue for twenty-four hours before guaiacum was added (benzidine was sometimes used and results were the same as with guaiacum). Light was then emitted in order to observe the reaction, and it was found that the plant tissues had turned blue on their surfaces, indicating the oxidation of guaiacum.

These experiments indicate that some process either in the decomposition of plants or in the bacterial action in connection with decaying plant tissues was producing an oxidizing agent capable of oxidizing guaiacum or benzidine. This reaction is independent of the presence of light and of molecular oxygen. Neither light nor detectable molecular oxygen are present at the

bottom of Lake Mendota where these plant fragments are abundant during the stagnation period. Consequently, this oxidizing agent must be present in plant tissues under such conditions. It would seem that this oxidizing substance was not something which was being stored, but was rather a substance which was continually being formed, inasmuch as plant fragments treated with guaiacum could later have the blue color washed out with alcohol and would again be capable of oxidizing fresh guaiacum.

The question again arises, is this substance which is found in the disintegrating plants and which is capable of oxidizing guaiacum atomic oxygen, or is it some other oxidizing agent? The following experiments, although they do not prove that this oxidizing substance is atomic oxygen, yet indicate that such is the case. The plant fragments found in the mud will oxidize guaiacum without the addition of hydrogen peroxide. Fragments boiled for a short period will not oxidize guaiacum unless hydrogen peroxide is added. As hydrogen peroxide has no effect on guaiacum, this seems to indicate that boiling destroyed a peroxide component of an enzyme complex in the plant tissues, for it did not destroy a substance capable of splitting atomic oxygen from hydrogen peroxide which then oxidized guaiacum. Active oxygen is the only oxidizing substance which can be split from hydrogen peroxide. It there foreseems probable that the enzyme-like substance, not killed by boiling, was in the unboiled plant fragments splitting off atomic oxygen from a naturally occurring peroxide. It was also shown by experiment that boiled plant tissues were capable of splitting off active oxygen from hydrogen peroxide and of oxidizing guaiacum in the absence of molecular oxygen and light.

Since the results were found to be constant regardless of when or where the experiment was performed, it follows that this oxidizing substance is constantly being liberated by the decaying plant remains on the bottom of Lake Mendota. If this oxidizing substance is atomic oxygen, then here is a source of oxygen for the animals which live there during the stagnated period. The amount of oxygen given off at any one time would be very small,

and would soon be taken up by decaying material and organisms, so would not remain long as such, consequently any animal utilizing the oxygen would have to be in close relationship with the plant remains. The burrows of chironomid larvae are regularly found to be made up of a considerable amount of bits of plant tissue, along with mud, and the whole cemented together by a secretion from the spinning glands. The larvae are therefore in a position to utilize any oxygen which might be produced.

DISCUSSION OF THE METABOLISM OF OTHER ANIMALS LIVING IN  
OXYGEN-FREE ENVIRONMENTS IN LAKE MENDOTA IN  
VIEW OF THE THEORIES SUGGESTED

This hypothesis is feasible as an explanation for the oxygen supply of all animals living in the stagnated region. It is applicable to the clam and the annelid worm whose enzyme content excludes the possibility of the production of oxygen by enzymatic action within their own bodies.

*Pisidium idahoense* Roper, the small clam which is regularly found in the oxygen-free water in Lake Mendota, does not have red blood. Neither does it contain an enzyme which is capable of oxidizing guaiacum without the addition of a peroxide. However, it contains a peroxidase-like enzyme which is not killed by boiling and which can split off atomic oxygen from a peroxide. The clam is inactive during the period of stagnation. Specimens kept alive in the laboratory for a period of over ninety days in bottles filled with a little bottom mud and oxygen-free water did not change their position during that time nor were they ever observed to have their valves open. Obviously, their inactivity, together with the low temperature, reduces their need of oxygen to the minimum. Living as they do in the mud, surrounded by the remains of plants, they would be able to utilize any small amounts of oxygen which might be given off by the plants, which would be sufficient to keep up the low rate of oxidation within their bodies.

Experiments similar to those made with the chironomid larvae were also conducted which showed that *Limnodrilus* sp. (?)

although it possesses red blood, apparently does not contain an enzyme complex capable of oxidizing guaiacum without the addition of a peroxide. This annelid has an iron-containing enzyme-like substance which is not killed by boiling and which can split atomic oxygen from a peroxide. It lives in tubes constructed of the mud and small bits of plants. In the laboratory it is also found among the tubes of chironomid larvae. Its association with plant remains would enable it to obtain from them any oxygen which might be liberated.

The larvae of *Sialis infumata* Newman, the black-winged orl fly, which are only occasionally found in the stagnated region in Lake Mendota, are very active. They are more or less predacious, crawling in and over the mud and preying upon such smaller organisms as they can find. They are also reported to eat mud containing organic material. *Sialis* larvae have no haemoglobin in their blood, and their tissues contain very little of the enzyme-like substance which requires a peroxide for the oxidation of guaiacum. This is quite different from the case of the chironomid larvae. However, the inner surface of the chitin of the *Sialis* larvae contains a thermolabile enzyme complex which will of itself, without the addition of a peroxide, oxidize guaiacum, but not so strongly as that in the chironomid larvae.

This second case of the presence of an enzyme complex which is capable of yielding an oxidizing substance—probably atomic oxygen—is additional evidence for the theory that at least a part of the oxygen supply of the more active animals living in water in which no dissolved oxygen can be demonstrated may come from a splitting off of oxygen from a previously built-up peroxide, the whole being due to enzymatic action.

No work was done on the protozoans, the gastrotrich, or the ostracod, which are also normally found living in the stagnated region of Lake Mendota.



## SUMMARY AND CONCLUSIONS

The results of the work thus far accomplished may be summarized as follows:

1. During certain periods of the year the water in the deeper parts of Lake Mendota contains no dissolved oxygen which can be detected. Nevertheless, several animals are able to lead a more or less active life in it and liberate energy for the maintenance of their normal physiological processes.

2. Theories as to the sources of their energy have been advanced. That the animals are facultative anaerobes, dependent upon oxygen from some source for the liberation of the potential energy stored up within their bodies, is highly improbable. It is thought that an incomplete breakdown of compounds, anoxybiosis, would not suffice to be an explanation of the source of the energy exhibited by many of the active animals.

3. As to the supply of the oxygen necessary to liberate energy, three theories have been set forth:

- a.* That oxygen is stored by the haemoglobin in times of oxygen plenty and then gradually used up in times of oxygen want, has been suggested. Not all the animals, in fact only a few, living in the stagnated region have haemoglobin; and even if they did, Leitch ('16) has demonstrated that the amount of oxygen which the haemoglobin is chemically able to fix and hold is negligible when compared with the amount necessary to liberate the energy expended during the total time when the dissolved oxygen is lacking in the surrounding water.

- b.* That the supply of oxygen is dependent upon the presence of an enzyme complex which is capable of building up a peroxide and then splitting off oxygen from it, is a possibility for some of the organisms at least. However, with the data at hand, this does not seem probable, inasmuch as the production of atomic oxygen could not be demonstrated when the conditions of the the experiments excluded the presence of molecular oxygen. Although further work may show that such a process furnishes an oxygen supply for certain animals, the theory makes no provision for other animals which live in the same environment

without oxygen, but do not possess the necessary enzyme complex for the performance of such a reaction.

c. That a process in the decomposition of plant tissues, even under anaerobic conditions, is gradually liberating small amounts of oxidizing substance had been demonstrated. That this oxidizing substance is atomic oxygen has not been absolutely proved, but experiments indicate that it is. If this oxidizing substance is atomic oxygen, then it can be utilized by all animals, living as they do in close relationship with the disintegrating plant fragments. The writer believes that the oxidizing substance produced by plant fragments is probably atomic oxygen and that it forms the oxygen supply of those animals living in Lake Mendota under conditions where the surrounding water shows no trace of dissolved oxygen.

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<sup>2</sup> Not seen by writer. Reference taken from Snyder ('12).

<sup>3</sup> Not seen by writer. Reference taken from Maill and Hammond ('00).

## APPENDIX

## DESCRIPTION OF APPARATUS AND METHODS EMPLOYED

*1. Method for determining the amount of dissolved oxygen*

The method for determining the amount of dissolved oxygen in the various samples of water tested, was the same as that employed by Birge and Juday in their work on the dissolved gases of inland lakes. This is known as the 'Winkler method' and is described in detail in their "Inland Lakes of Wisconsin. The Dissolved Gases of the Water and their Biological Significance ('11)." It depends primarily on a change of manganous hydroxide to manganic hydroxide by taking up the oxygen dissolved in the water. The manganic hydroxide reacts with hydrochloric acid, and chlorine is liberated, which in turn reacts with potassium iodide, liberating iodine. The iodine liberated is directly proportional to the amount of dissolved oxygen which the water contained. The iodine can be titrated against standardized sodium thiosulphate, using starch as an indicator. The results, in terms of cubic centimeters of oxygen per liter, can be computed by use of a given formula.

This method is the simplest and most satisfactory of the various methods for oxygen determination and was compared by Birge and Juday with the boiling method, which they also used, and found to check within the limits of error.

*2. Methods used in obtaining oxygen-free water from the lake bottom*

The apparatus employed in obtaining samples of water from the stagnated area was borrowed from the Wisconsin Geological and Natural History Survey. The pump has been described and figured by Juday ('16). It consists of a hand pump with a hose attachment. A calibrated line was fastened to the end of the hose so that the intake could be regulated to any desired depth. The exit tube from the pump was connected with a short glass tube which could be inserted to the bottom of the collecting bottle. By pumping a large amount of water through the bottle and then gradually removing the tube, water was obtained from any depth in the lake without coming in contact with the upper water or with the air. The bottles used had an average capacity of about 250 cc. and were provided with a sealing attachment which insured a tight stopper in the mouth of the bottle. By filling the bottle level full and inserting the stopper obliquely, thus forcing out some of the water at the surface, a sample of water was obtained in which there were no bubbles of air. Samples taken in this manner and tested by the Winkler method for oxygen were used in determining the extent of the stagnated area in Lake Mendota.

By lowering the intake hose to the surface of the mud, water containing mud could be pumped up. The mud was used after settling



as food for the chironomid larvae or other organisms, which were either introduced into the bottle before sealing or pumped up with the mud. Bottles containing water and living organisms taken from the stagnated region which were to be used for observation in the laboratory were inverted and their necks dipped in melted paraffin thus doubly sealing them.

### *3. Methods for observation of animals under normal conditions*

Series of samples in bottles, obtained in the manner described in section 2 of this appendix were kept in an underground cellar in darkness and at a temperature of 4.5° to 8°C. The confined organisms were as near normal condition as possible and still capable of being observed. The only method that could be employed of determining the habits of the animals studied under normal conditions was by the observation of individuals confined in the bottles containing oxygen-free water and mud and kept in the manner just described.

### *4. Method employed for collecting animals for experimental purposes*

Animals living in the mud at the bottom of Lake Mendota were collected for experimental work by means of a mud-dredge. This apparatus is similar to the Ekman ('15) dredge. It consists of a brass box with movable jaws which form the bottom. These jaws are held together by springs. They can be pulled up at the sides and fastened. When the dredge is lowered to the bottom from the boat or from the ice by means of a line, its weight carries it down into the soft mud. The catch holding the jaws apart can then be released by a messenger and the springs pull the jaws together, thus enclosing in the dredge a quantity of the mud with the organisms living in it. The dredge is drawn to the surface, its contents poured into a sieve, and the animals washed free from the mud.

### *5. Method for making water oxygen-free and technique for handling it*

The apparatus shown in figure 1 was constructed during the spring of 1917, being completed before the writer learned of a similar apparatus used by Pause ('18). It provided for the boiling of water until all the oxygen was driven off, the presence of oxygen being detected by the Winkler method.

The apparatus consisted of a 5-liter flask (*A*) in which water was boiled for a long period of time, thus driving off the dissolved oxygen. A metal stopcock (*D*), which provided an opening to the flask, was closed when the water stopped boiling, thus cutting off all connection of the water with the outside. Nitrogen was then introduced from the tank (*N*) through the tube *I*, to keep the pressure on the inside of the flask, which would be lowered on cooling, equal to the atmosphere pressure. The pressure on the inside was indicated by the movement of the water in the tube *G*.

The tank of commercial nitrogen used contained a small amount of oxygen, as was determined by means of the Hemple gas pipette. The oxygen was removed by passing the gas through two bottles of alkaline pyrogallol ( $P_1$ — $P_2$ ).

Flasks of 150-cc. capacity were used in order to observe the reactions of the organisms studied in the deoxygenated water. Each observation flask ( $F$ ) was provided with two tubes which were inserted through the stopper. The intake tube was longer, reaching nearly to the bottom of the flask, while the exit tube was level with the stopper. Both tubes were provided with stopcocks, which were closed while the experiment was in progress.

To fill the observation flask ( $F$ ) with deoxygenated water without contact with air, the flask was first filled with tap water, its intake tube connected with the exit tube of the large flask ( $A$ ), and the observation flask inverted. Nitrogen was then led from the tank ( $N$ ), purified in the pyrogallol bottles ( $P_1$ — $P_2$ ), through the tube ( $C$ ) passing around the boiling flask ( $A$ ), and thence into the observation flask ( $F$ ), where it drove out the water. Following this the flask ( $F$ ) was righted, the stopcock on tube  $C$  was closed, and the stopcocks on tubes  $I$  and  $E$  opened. Nitrogen was then passed through tube  $I$  to the bottom of the boiling flask ( $A$ ) thus stirring up any mud which might have been boiled with the water. The pressure forced the oxygen-free mud and water out of the flask through the tube  $E$  and into the observation flask ( $F$ ) where it displaced the contained nitrogen. Organisms to be observed in the oxygen-free water were placed in the observation flask before the filling process began, care being taken that they were not washed out during the filling.

As soon as one observation flask was filled, it was disconnected from tube  $E$  and another could be put in its place. Series of flasks containing deoxygenated water and organisms were sealed with paraffin or asphaltum and kept in the cellar where light and temperature factors were as close as possible to those under normal conditions at the bottom of Lake Mendota.

#### *6. Methods used in experimental work on animals*

The guaiacum used was freshly prepared 1 per cent solution of gum guaiacum in 95 per cent alcohol. The benzidine solution was made by dissolving 3 to 4 mgr. of benzidine crystals in 2 cc. of glacial acetic acid. Commercial hydrogen peroxide was used.

Aqueous extracts of animals were made by grinding them in a watch crystal with a pestle.

To determine whether or not the enzyme complex which was found in the chironomid larvae would oxidize guaiacum in the absence of molecular oxygen, the writer constructed the apparatus shown in figure 2. This apparatus provided for the removal of oxygen from an experimental chamber, in which reactions were then allowed to take place.

The apparatus consisted of a bell jar (*A*) inserted in the jar *B*, which was partially filled with water. Jar *A* was connected at its top with a gas (commercial nitrogen or carbon dioxide) tank (*D*). Suspended from the top of jar (*A*) were two wire baskets (*C*), containing yellow phosphorus, which would remove any oxygen in the gas withdrawn from the tank (*D*) and collected in jar (*A*) over the water in jar (*B*). Pyrogallol could not be used to remove the oxygen on account of the fact that carbon dioxide was used for some of the experiments.

Samples of gas could be withdrawn from jar *A* into the collecting tube *F* of a Hemple gas pipette (*P*), which was used to measure the amount of oxygen present. When the phosphorus had removed all the oxygen from the gas in the jar *A*, the gas could then be led out through the tube *E*, into the small bottle *G*. This bottle contained water in which cuprous chloride was dissolved. The water took out phosphorus pentoxide fumes, formed by the union of phosphorus and oxygen in jar *A*, and the cuprous chloride took up any oxygen which the water might contain, being changed in this process to cupric chloride. From jar *G* the deoxygenated gas could be led through tube *I* into one side of an H-shaped experimental chamber (*K*). The connection of the two tubes of this chamber was about 1 cm. from the lower end. A pocket was thus formed at the bottom of each tube. Tube *J* led from the other side of the H-shaped chamber to the exterior and opened beneath the surface of water, being thus sealed.

The aqueous extract of the organism, and the indicator (guaiacum or benzidine) could be placed in separate pockets of the chamber. The deoxygenated gas could be then led through the chamber (rather rapidly at first) which would wash out all the air. Later, the flow could be decreased and continued for any desired length of time, thus removing by diffusion any oxygen dissolved in either the extract or the indicator. It was assumed that all the oxygen would be removed by this method after a continuous flow of the deoxygenated gas of from two to three hours. At the end of that time the chamber could be tipped on a horizontal axis and the indicator poured into the solution to be tested. In this way a reaction could be obtained in the absence of molecular oxygen.

In order that an aqueous extract of an organism could be made in the absence of oxygen, the experimental tube was provided with a grinding apparatus, which consisted of a glass pestle (*N*) working through a stuffing box (*O*) at the upper end of the chamber against small pieces of broken glass in the bottom of the tube. Extracts of organisms could thus be made in any stage of an experiment.

This same apparatus (fig. 2) was used in the experiments with plant remains found in the mud at the bottom of the lake, except that the experimental chamber was inclosed with a covering of black paper which shut out the light and thus insured against the production of oxygen by a photosynthetic process.

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Resumen por el autor, H. H. Newman  
Hopkins Marine Station of Leland Stanford University  
and Hull Zoological Laboratory of the University of Chicago.

La producción experimental de gemelos y mónstruos dobles en las larvas de la estrella de mar *Patiria miniata*, junto con una discusión de las causas productoras de gemelos en general.

El autor ha conseguido producir una serie de gemelos separados y monstrosos dobles bajo tres diferentes condiciones experimentales: (a) Como resultado de un desarrollo partenogenético extremadamente retardado; (b) como resultado de la fecundación de los huevos de *Patiria* por los espermatozoides de otras estrellas de mar; (c) como resultado de la aglomeración de óvulos fecundados normalmente. Los factores comunes a los tres métodos suponen: (a) Retardamiento del desarrollo con pérdida de la organización axiada tan precisa durante algún periodo crítico. (b) Rediferenciación o nueva adopción de la organización axiada, habiéndose perdido la unidad de organización de tal modo que en vez de aparecer un solo eje o gradiente aparecen dos o más. De este modo se originan gemelos o estructuras dobles.

Se puede producir una serie de tipos de gemelos que representan los resultados de las diferencias en la época del comienzo del retardamiento y regeneración más o menos completa. La serie incluye blástulas y gástrulas que presentan la mitad y cuarta parte del tamaño normal, gástrulas de tamaño normal con dos o más arquenterios, larvas en las cuales el arquenterio está dividido anteriormente en larvas "dicéfalas," y bipinarias en periodo avanzado del desarrollo con poros madreporicos y canales poricos pares en vez de un solo poro a la izquierda. La teoría fisiológica de la producción de gemelos está de acuerdo con la expuesta anteriormente por el autor para explicar la causa de la poliembrionía específica del armadillo.

Translation by José F. Nonidez  
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# THE EXPERIMENTAL PRODUCTION OF TWINS AND DOUBLE MONSTERS IN THE LARVAE OF THE STARFISH PATIRIA MINIATA, TOGETHER WITH A DISCUSSION OF THE CAUSES OF TWINNING IN GENERAL

H. H. NEWMAN

*Hopkins Marine Station of Leland Stanford University and the Hull Zoological  
Laboratory of the University of Chicago*

FORTY-SIX FIGURES

## THE PROBLEM STATED

A great deal has been written about twins during the last decade and much interest has been shown in the general biological aspects of twinning, especially in the bearing of twinning on sex determination and sex differentiation, on the limits of hereditary control, on symmetry reversal, and on the heredity of the twinning tendency. Little attention, however, has been paid to the physiology of twinning: the causal factors responsible for the doubling of normally single individuals or structures. It is the function of the present paper to throw some light upon this obscure problem.

When the writer first became interested in the biology of twins, the problem of the physiological basis of twinning assumed a different aspect from that which now presents itself. The production of two individuals or parts, where one would normally appear, was at first thought a supernormal process—a developmental excess, due presumably to some extraneous stimulus. It soon became clear, however, that this naive first conjecture was incorrect, for several lines of evidence seemed to point to the diametrically opposite conclusion: that twinning is a subnormal phenomenon associated with a depressed or retarded

developmental condition. Seven years ago the writer published a theory purporting to explain on a physiological basis the extraordinary phenomenon of specific polyembryony in the nine-banded armadillo (*Dasypus novemcinctus*). The tenor of this theory was that the armadillo egg became temporarily retarded so as to cause a slowing down of its metabolic rate or intensity and a consequent loss of its axiate structure; that when development was resumed four centers of differentiation arose instead of the original one, and quadruplet embryos were the result. Subsequently Patterson discovered that the armadillo blastodermic vesicle, in a stage equivalent to a gastrula, lies quiescent in the fallopian tubes or in the uterine cavity for a period of perhaps three months. On the basis of this observation, the writer, in his book on *The Biology of Twins*, formulated the following theory:

In the armadillo egg the ectodermic vesicle has an apical point, which is the head end or growing tip of the embryo before the process of fission (polyembryony) occurs. If the conditions of growth were such as to admit of a normal rate of metabolism, this original apical end would become the head end of a single embryo. Some agency lowers the rate of metabolism of the embryo and the original apical end loses its dominance over subordinate regions; the result is that several radially arranged secondary points in the ectodermic vesicle acquire independence. Those that are most favorably situated with reference to the uterine axes express their independence first and become the first visible growing points, the so-called 'primary buds;' those that are less favorably situated acquire independence later and form the so-called 'secondary buds.' It happens that, almost synchronously with the physiological isolation of the whirl of subordinate growing points, a new and effective nutritive connection (the Träger ring) is established between the embryonic vesicle and the maternal tissues, which greatly accelerates the metabolic rate and the consequent speed of growth. This rejuvenating factor stops the production of further growing points and makes it possible for each of the newly formed apical ends (heads) to develop a body. When the conditions of growth are restored to normal, the vesicle is no longer a single individual, but is a clone, consisting of four essentially separate individuals, each of which goes through its own embryonic development quite independently of the others, except in so far as development within a common chorion and the necessity of sharing a single primary placenta involve mutual adjustments.

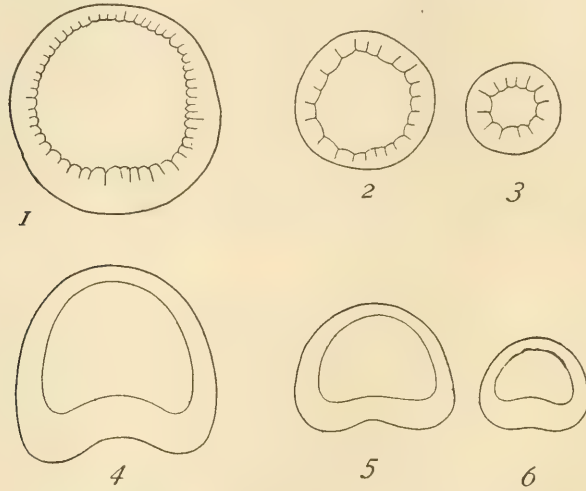
This theory met with a degree of coolness and skepticism, partly, no doubt, owing to the fact that it was based upon Child's 'axial gradient theory,' a far-reaching and consistent principle, which, though at first coldly received or ignored by American biologists, is slowly but surely coming into its own. It has been the writer's desire for some years past to test out this physiological theory of twinning experimentally. The armadillo material is too difficult to secure or to manage. It is therefore advisable to work with some more available material. When making plans for a period of several months' work at Pacific Grove, California, the writer hoped to encounter some marine material that would be suitable for twinning experiments, and was fortunate to find in the starfish *Patiria miniata* a species almost ideal for this purpose.

#### MATERIAL AND METHODS

*Patiria miniata* is a common starfish of the California coast. It belongs to the first order of the subclass Asteroidea, viz., Spinulosa. This is the same species which Loeb used for experiments on artificial parthenogenesis and to which he applied the name *Asterina*. For most purposes *Patiria* is a decidedly unfavorable species, because it is so difficult to obtain eggs in prime or uniform condition. Attempts to cause the extrusion of ripe eggs from the genital pores have been unavailing, and the somewhat crude method of gently shaking the carefully excised ovaries in sterile sea-water has been adopted as a means of obtaining eggs for experiment. For most purposes this method furnishes too large a percentage of immature eggs and too wide a range of diversity in the state of ripeness of the full-grown oöcytes. Of the eggs that appear to be fully mature, a large though varying per cent never undergo maturation; some that mature form membranes simulating fertilization membranes, but do not undergo parthenogenetic cleavage; usually a small percentage of eggs that mature and do not form a membrane undergo parthenogenetic cleavage; while the majority of the matured eggs neither form a membrane nor undergo cleavage unless fertilized either by sperm of the same species or by that



of some other species. It will be seen, then, that the material is somewhat complex and difficult to control by means of exact experimental manipulations. We are therefore forced to make use of certain natural experiments which fortunately have come to hand and which appear to furnish the data needed for an analysis of the causal factors involved in twinning.



(Figs. 1 to 28 are from parthenogenetic cultures)

Fig. 1 Normal blastula, showing distinct polarity, small cells at apical end, large cells at basal.

Fig. 2 Half-sized dwarf blastula with indefinite polarity.

Fig. 3 Fourth-sized dwarf blastula with little or no polarity.

Fig. 4 Normal early gastrula.

Fig. 5 Rare case of typical half-sized gastrula, resulting from half-blastula that had acquired a definite unipolar axis.

Fig. 6 Rare case of quarter-sized gastrula from a polarized quarter-sized blastula.

The first evidences of twinning that came to the writer's attention appeared in certain hybrid strains in which the eggs of *Patiria* had been fertilized by the sperm of another asteroid, *Pisaster ochraceus*. In several cultures of these cross-bred strains there were noticed, on the second day, considerable numbers of very small blastulae and gastrulae, some about one-half and others one-fourth as large as normal larvae at the same stages. A good idea of the size and appearance of some of the more

nearly normal types of these dwarf larvae may be gotten from figures 2, 3, 5, and 6, as compared with the normal (figs. 1 and 4),

These larvae were too small to be interpreted as retarded larvae, for they were less in diameter than the unsegmented egg. Neither could they be due to chance admixtures of larvae from another species. It was, therefore, assumed as a working hypothesis that they were twin larvae derived by the physiological isolation of the blastomeres of the two-cell and four-cell stages. This assumption was substantiated by a detailed study of cleavage and early development. During this study there came to light several other types of twins, and these proved more significant than those first mentioned. As the work extended, it developed that twins of one or more types appeared under three distinct conditions: *a*) as the result of the spontaneous parthenogenesis of a small percentage of eggs; *b*) as the result of crossing *Patiria* eggs with the sperm of other echinoderm species, and, *c*) as the result of crowding the normally fertilized eggs of *Patiria*. It is now proposed to present separately the data derived from each of these types of experiment.

The illustrations for this paper represent camera drawings of quieted individuals. Only structures that are primarily of interest in connection with twinning are shown.

#### A. TWINS IN PARTHENOGENETIC CULTURES

As was brought out in a recent paper (Newman, '20 a), some in practically every lot of *Patiria* eggs, in which every precaution has been taken to avoid accidental fertilization, undergo spontaneous parthenogenetic development. The percentage of matured eggs that at least begin cleavage under these conditions varies from 0 in an occasional culture to about 75 in one culture. The usual number ranges from 1 to 10 per cent and is most commonly 2 or 3 per cent. These eggs that are destined to develop parthenogenetically undergo maturation, as may be determined by the disappearance of the germinal vesicle within an hour or two after the eggs are shed into sterile sea-water. After maturation nothing further happens for from five and one-half to six hours, following which lapse of time the eggs, without

forming any fertilization membrane, begin cleavage. In normally fertilized eggs cleavage begins within about two hours after maturation, if insemination occurs at the time when the majority of the eggs have undergone maturation. The parthenogenetic eggs are therefore about three hours slower to begin cleavage than are the normally fertilized eggs. This very pronounced retardation involves a period at the very beginning of ontogeny, when any serious retardation must of necessity exert a telling influence upon subsequent development. The effects of this influence vary in severity in different eggs, presumably in accord with variations in their physiological condition. In the following paragraphs we shall deal with a considerable list of observed conditions, which are arranged in the order of success in development, beginning with cases in which the effects of early retardation have been most pronounced and ending with those cases in which the larvae show the most complete recovery from an early inhibition.

*a.* Some eggs begin, but are unable to complete, the first cleavage. It is quite common to find eggs with two nuclei and a cleavage furrow about half-way across the egg. Such eggs when kept under observation for several hours show no change and undergo cytolysis within the next twelve hours. In this case the eggs evidently have been so profoundly inhibited that only a spark of developmental energy remains—a spark which flares up momentarily, but dies out before the first cleavage is complete.

*b.* Other cases were observed in which, after the successful completion of the first cleavage, one blastomere only was able to continue cleavage. The other blastomere remains for many hours uncleaved and is sometimes partially surrounded by the cells resulting from the cleavage of the other cell. This looks somewhat like a case of epibolic gastrulation. Occasionally the cells of the cleaving blastomere round up into a sort of solid dwarf blastula and become released from the vitelline membrane as a swimming larva. This has been observed several times, and was especially interesting in those cases where such an active larva has had to tow about through the water the inert uncleaved blastomere, which has been invaded by parasitic protozoa.



Figure 11 shows a case in which a nearly normal blastula is formed from one blastomere while the other blastomere has remained uncleaved. The ability of one blastomere to undergo cleavage while the other lacks this ability suggests that the products of the first cleavage are physiologically different. It is well known that the first cleavage in echinoderms divides the egg into the prospective right and left halves of the body. In the cases under discussion one side is evidently endowed with a greater degree of developmental energy than the other, and this may be an early indication of the characteristic unilaterality in development that subsequently expressed itself in the asymmetrical development of the two sides prior to metamorphosis, and the consequent change from bilateral to radial symmetry. This is perhaps a far-fetched conjecture, but other data subsequently brought out serve to lend it considerable support. A good many interesting conditions were noted in early cleavage stages. In some cases cleavage was evidently proceeding separately in the case of the derivatives of the first two blastomeres. A very clear case is shown in figure 7, which shows the cleavage of one blastomere in a completed four-cell stage and that of the other blastomere in a two-cell stage. Such an early condition probably results in twin blastulae only slightly different, as in figure 8. Other cases occur, as in figure 9, in which the products of cleavage of one blastomere are in a normal four-cell stage, while those of the other blastomere are irregular and are showing signs of disorganization. Almost every kind of isolation of groups of blastomeres is to be noted, and it is not uncommon to find minute balls of ciliated cells attached to an unorganized mass of large cells. A great many instances of exogastrulation occur, but these do not concern us in this place.

c. One of the first evidences of twinning in *Patiria* was based on the discovery of numerous dwarf larvae, chiefly blastulae, which were only one-half or one-fourth the normal size (figs. 2, 3, 5, 6). At the time of this first discovery there was no evidence to confirm the suspicion that these larvae had originated by a process of blastotomy. Subsequently, however, the confirmatory evidence appeared, and now we feel safe in concluding that



wherever half-sized and quarter-sized blastulae and gastrulae occur they are to be regarded as the products of the physiological isolation of the blastomeres of the two-cell or of the four-cell stages. As has already been pointed out, some of the eggs in a culture, after completing the first or the second cleavage, come to rest for a period of several hours, and when cleavage is again resumed, each blastomere behaves as though it were a complete ovum and forms a complete half-sized or quarter-sized blastula.

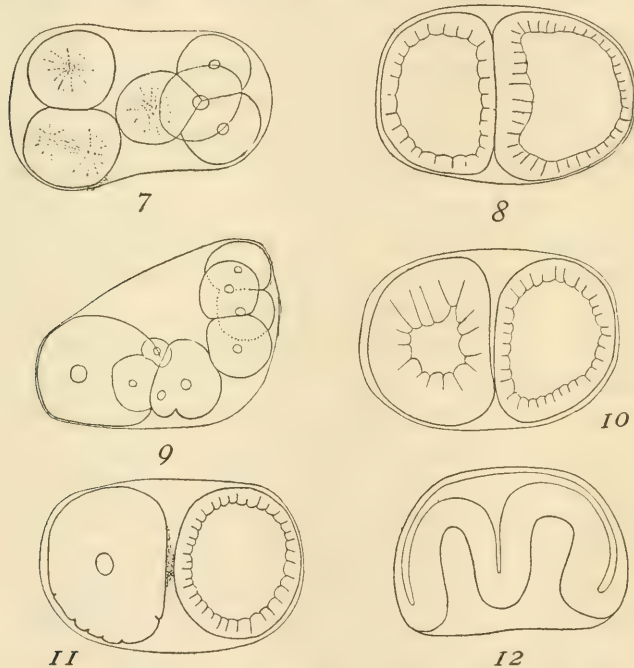


Fig. 7 A good case of the early isolation of the blastomeres in the two-cell stage and the subsequent independent cleavage of the two cells.

Fig. 8 Twin blastulae within one vitelline membrane, probably derived from a condition like that shown in figure 7.

Fig. 9 A case of isolation of the blastomeres of the two-cell stage and its consequences: a good four-cell stage and an irregular cleavage.

Fig. 10 Twin blastulae, one of which is much more advanced than the other.

Fig. 11 A case in which only one of the first two blastomeres has undergone cleavage. This has formed a blastula with very little axiate organization.

Fig. 12 Rare case of twin gastrulae, probably resulting from only partial isolation of the cleavage products of the first two blastomeres.

It is not often that one finds four complete blastulae surrounded by a single vitelline membrane, but it was possible to make camera drawings of two such cases. Specimens showing two blastulae within a single membrane (figs. 8 and 10) are, however, relatively common, and in one instance there was observed the rupture of the membrane and the total separation of the twin blastulae. One fact that numerous drawings show, although at first no particular significance was attached to it, is that the two blastulae are seldom, if ever, alike. One of them seems to be less advanced, or at least less normal, than the other. In some cases, for example, one blastula is composed of smaller and more numerous cells and has a larger cavity than the other. In other cases one blastula is quite normal while the other is a solid blastula, in which the lumen is filled with cells and the peripheral cells are longer than normal. These observations tend to support the conclusion, mentioned in a previous paragraph, that the later bilateral asymmetry characteristic of echinoderm larvae is pre-determined physiologically in the two-cell stage or even prior to cleavage. If this contention is well founded, it might be possible in the unsegmented egg to demonstrate for echinoderm eggs, as Conklin has demonstrated for the eggs of dextrally and sinistrally spiral gastropods, a structural asymmetry of the cytoplasm. Even if no visible structural asymmetry exists, there is good evidence in these experiments that a physiological asymmetry is present which, when exaggerated by inhibiting factors, as is the case in the unequal twins just described, makes itself clearly evident.

The fate of these dwarf blastulae has not been followed out with entire satisfaction, but it seems to be safe to say that only the more normal of the twin blastulae ever gastrulate. A large number of dwarf gastrulae have been noted, some occurring in almost every lot of larvae examined, but there seem to be no indications that these dwarfs progress beyond an early complete gastrula stage. If they do they have escaped attention.

The cases described in this section hitherto have dealt with more or less completely isolated twin blastulae, but there are frequent instances of incomplete isolation of the twins and the

production of double monsters, such as Siamese-twin blastulae or gastrulae. In most cases these double embryos consist of two blastulae of unequal size which together constitute a dumb-bell-shaped mass with two cleavage cavities. In one case (fig. 12) there was found a double larva in which gastrulation had occurred equally well in both the right and the left component. It is fair to conclude that these are instances of a later or less complete isolation of the original blastomeres of the two-cell stage, bearing the same relationship to the completely separate twins that human double monsters do to human duplicate twins.

*d.* The individuals in which cleavage goes on typically, though at a rate slower than normal, reach the blastula stage without further evidences of twinning, but one who has studied the fate of a sufficiently large number of such blastulae learns to differentiate them into several groups. The first group consists of solid blastulae in which the absence of a lumen renders embolic gastrulation impossible. Whether gastrulation by delamination or by the inward migration of endoderm cells takes place has not been determined. There is no evidence that solid blastulae ever progress beyond that stage. The second type, which is quite common, is one in which the blastula is without visible polarity. All of the cells are of equal size and there is no indication of a distinct animal and vegetal pole (fig. 13; cf. fig. 1, a normal blastula). Such blastulae undergo multiple gastrulation. The surface invaginates in numerous places and a bizarre type of larva (fig. 14), all wrinkles and pockets, appears, which may live for days unchanged. A third type, or really series of types, occurs in which a double polarity is evident. Instead of only one thickened plate of endodermal cells there may be two (fig. 15) or even, in rare case, three (fig. 17) such plates, and gastrulation occurs at two (fig. 16) or three (fig. 13) places. The two plates may be closely conjoined on one side of the blastula; they may be directly opposite to each other or they may have axes at right angles to each other. The result is that double, occasionally triple, larvae are formed in which there is but one ovoid larval body, but two, three, or more archentera. As a rule, there is no difficulty in distinguishing a primary archenteron

and one or more secondary archentera. If we use figure 19 as a norm representing a typical gastrula, we may note the various types of larvae with plural archentera. Figure 23 shows an almost normal larva, atypical only in the presence of a second thickened region at the apical end of the larva. Figure 24 shows a common

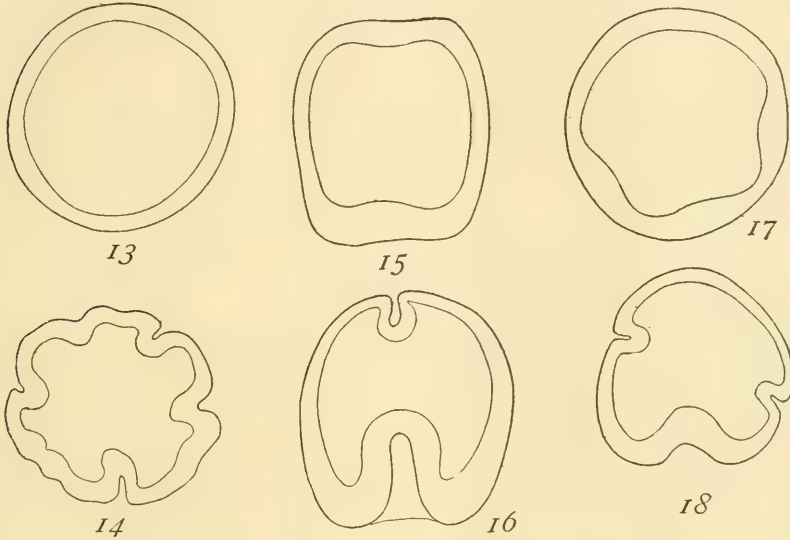


Fig. 13 Outline drawing of a blastula without axiate organization.

Fig. 14 A gastrula with multiple archentera derived from a blastula like that in figure 13.

Fig. 15 Blastula with two basal regions, a primary and a secondary.

Fig. 16 A gastrula with two archentera, the secondary forming at the apical end.

Fig. 17 A blastula with three basal areas.

Fig. 18 A gastrula with a primary and two secondary archentera resulting from a larva like that in figure 17.

condition in which this thickened apical plate has undergone a slight, but definite invagination. Figure 21 shows a larva with three small, but very distinct, secondary archentera at the apical end of the larva. Figure 22 represents a much more distorted type of twin larva in which the secondary archentera, one on the side and one at the apical end, are large, though considerably smaller than the primary archenteron. Figure 20 is a rare type



in which the primary archenteron has evidently undergone very early fission, and there is, in addition, a small secondary archenteron at the apical end. There have been seen in parthenogenetic cultures not a few cases in which paired archentera arise in such a way as to produce symmetrical conjoined twins. In

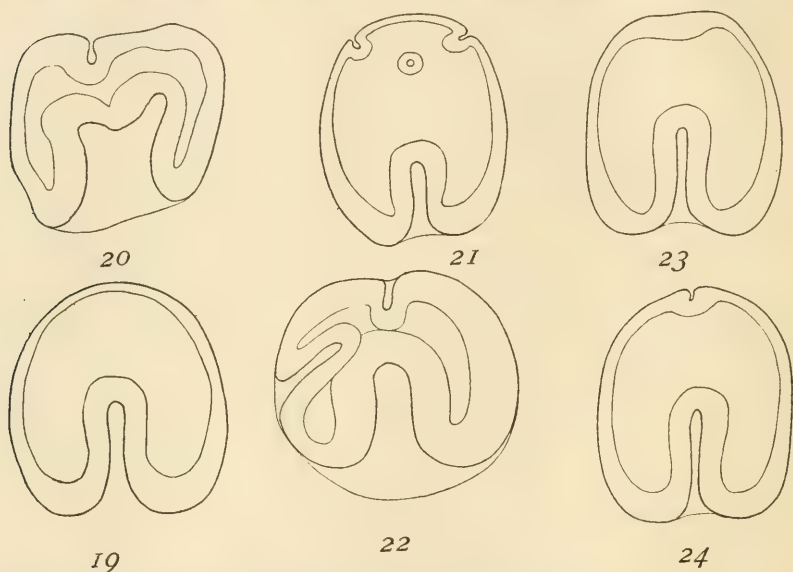


Fig. 19 A typical gastrula about forty-eight hours old.

Fig. 20 A very abnormal twin gastrula with paired primary archenteron and a small secondary archenteron at the apical pole.

Fig. 21 Gastrula with normal primary archenteron and three small secondary archentera near the apical end.

Fig. 22 Very irregular gastrula with three grades of archentera.

Fig. 23 A type of larva with an 'apical plate' which is merely a secondary basal region in which gastrulation has not yet taken place.

Fig. 24 A type of larva doubtless resulting from a condition like that shown in figure 23, in which a minimal secondary gastrulation has occurred.

these cases it seems evident that the formation of a primary archenteron, unlike the conditions previously described, has been inhibited and secondary archentera arise at equal distances from the site of the original basal end of the blastula. An interesting series of these mirror-image conjoined twins is shown in figures 25 to 28. Figure 25 shows a larva with two archentera both

near the basal end, and of equivalent size. Such larvae as this live for days unchanged. Figures 26, 27, and 28 show three conjoined twin larvae in which the paired archentera are formed nearly at right angles to the original primary axis. In the larva shown in figure 28 both archentera have taken the initial steps in the development of the hydro-enterocoel vesicles, but the process was never actually accomplished.

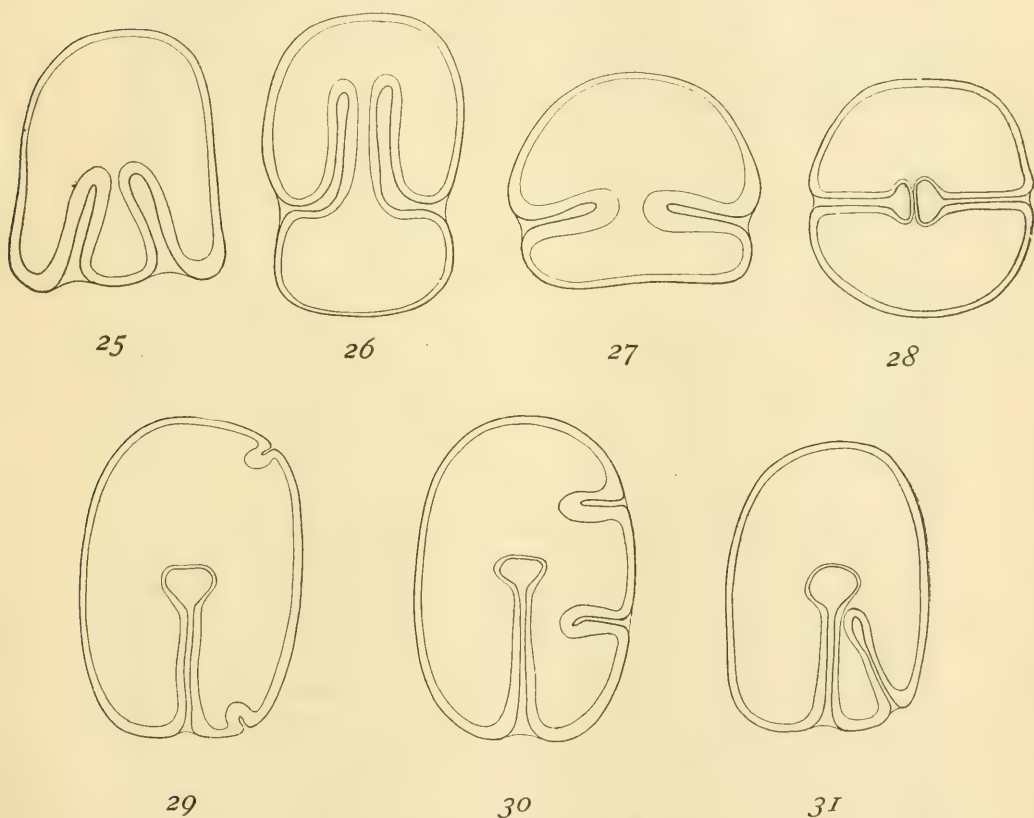


Fig. 25 A symmetrical twin larva with both archentera growing in near the original basal end.

Figs. 26, 27, and 28 Types of symmetrical twin larvae with archentera growing in from opposite sides of the larva. These are all mirror-image duplicates.

(Figs. 29 to 37 are from hybrid cultures)

Figs. 29, 30, and 31 Relatively large active larvae with well-differentiated primary archenteron, but comparatively poorly developed secondary archentera.

*Summary of data on twinning in parthenogenetic cultures*

All of the larvae derived from parthenogenetic *Patiria* eggs are to be viewed as decidedly subnormal. They show many evidences of profound inhibition. A whole graded series of inhibition products are found, including abortive first-cleavage stages; cases in which one blastomere of the two-cell stage segments and the other does not; cases in which the two blastomeres become physiologically isolated and produce conjoined or entirely separate twin blastulae, and later conjoined or separate twin gastrulae; cases of blastulae which have no polarity and undergo multiple gastrulation, and cases of bipolar blastulae that produce several distinct types of double-monster gastrulae with two or more archentera. Never do larvae from parthenogenetic eggs reach a true bipinnaria stage. The larvae are always deficient in the apical or anterior regions. There is usually a deficiency in the size of the preoral lobe of the larva, and there is seldom, if ever, any development of hydro-enterocoel pouches at the anterior end of the archenteron. In a very few cases larvae have been reared somewhat further than those figured.

## B. TWINS IN HYBRID PATIRIA LARVAE

When the eggs of *Patiria* are fertilized in normal sea-water by means of the sperm of another starfish, *Pisaster ochraceus*, a very wide range of success in development is observed. In addition to all of the types described for parthenogenetic eggs, there occur various other more advanced types of twin larvae and a whole series of more or less normal single larvae. Not infrequently there occur larvae in these hybrid strains that appear to be exactly like the advanced bipinnaria of the maternal species. It is very probable, though not certain, that most of the severely inhibited larvae come from eggs that have developed parthenogenically. This statement is supported by the fact that even in normally fertilized cultures of *Patiria* eggs there are found eggs that behave exactly like those in parthenogenetic cultures. The fact that a large proportion of the larvae succeed markedly better in hybrid cultures than in parthenogenetic cultures makes

it certain that true fertilization occurs in a large percentage of the matured eggs. Moreover, it is easily possible to note that all of those eggs that form a fertilization membrane and begin cleavage continue their development to relatively advanced stages. The principal difference between pure-bred and hybrid *Patiria* eggs or larvae, is one of rate of development; for the hybrid larvae from early cleavage stages on, are distinctly retarded as compared with the pure-bred. Statistical counts of two-, four-, eight-, sixteen-cell stages in pure-bred and in hybrid stocks, fertilized from the same lot of eggs and at the same time, showed a very striking difference in degree of advancement. In one experiment, for example, when the pure-bred stock showed a great majority of eight- and sixteen-cell stages, the hybrid stock showed not a single egg with eight cells and a majority of two-cell stages. A few hours later the difference was even more striking. One cannot escape the conclusion, therefore, that the developmental rate of the hybrid strains is distinctly slower than that of the pure-bred strains, and that the foreign sperm has exercised, in addition to its development-initiating function, a development-retarding action. Whether this effect is due to malcoordination or to some toxic influence matters little for our discussion. We are at least certain that there has been an early and more or less pronounced inhibition, which is more or less completely recovered from in the later stages of development. This inhibition is less profound, however, and is more completely recovered from than is the case in parthenogenetic eggs. Exclusive of twins adjudged to be the product of parthenogenetic eggs, we find in hybrid *Patiria* cultures very pretty examples of the more advanced types of larvae with double, triple, and multiple archentera. Especially common is the type in which a smaller secondary archenteron occurs at or near the anterior end of the larva. A considerable number of such double larvae have been seen to develop the ciliated bands characteristic of the bipinnaria larva. A good series of the more advanced type of double larvae in hybrid cultures is shown in figures 29 to 32. It will be noted that in most cases only the larger or primary archenteron undergoes any differen-



tiation. In many instances the hydro-enterocoel pouches were well on their way toward being pinched off from the anterior part of the primary archenteron, but no such changes occurred in the secondary archenteron. An interesting case is shown in which an advanced larva has two small supernumerary archentera, one near the anterior end and the other near the posterior end (fig. 29). Another case is shown in figure 30, in which two fairly large secondary archentera have grown in at right angles

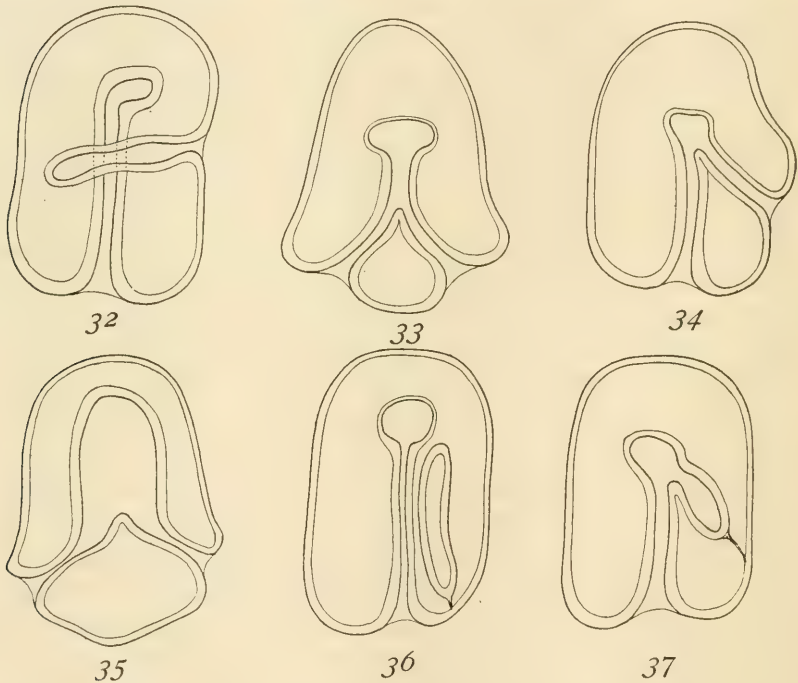


Fig. 32 A somewhat rare type of larva in which the primary and secondary archentera, though at right angles to each other, are nearly of equal value.

Figs. 33 and 35 Types of symmetrical twin larvae in which paired, symmetrically placed archentera have fused to form a common 'head end' of the archenteron.

Fig. 34 A case of the fusion of anterior ends of two nearly equal archentera asymmetrically placed.

Fig. 36 An instance of the closing off of a large secondary archenteron in the attempt to regulate back to the 'single' condition.

Fig. 37 The usual result of fusions like that in figure 34. This is another method of regulation adopted by asymmetrical double larvae.

to the primary archenteron. A common type of larva is shown in figure 31, in which the secondary archenteron is near the posterior end, but is distinctly less advanced than the primary. The extreme case of successful growth of an asymmetrical secondary archenteron is shown in figure 32, where this structure is nearly on a par with the primary, though coming in at right angles to the main axis of the larva.

*The results of secondary fusion of archentera*

A great many cases have been noted in hybrid cultures, though occasionally found both in parthenogenetic and in normally fertilized cultures, of conjoined twins produced by the fusion of the secondary archenteron with the primary. A case like that shown in figure 34 is probably due to the fusion of archentera in a larva that started out much like that in figure 32. It is also probable that the larva shown in figure 37 is a more advanced condition of the same larva that is shown in figure 34, since it was found two days later in the same culture. Many other cases have been noted in which there is a tendency for regulation to take place in such twins, involving an absorption of the secondary archenteron by the primary, and a return to a single, almost normal individual. An account of this process, though interesting from other standpoints, is scarcely germane to the present discussion, and is therefore postponed for a subsequent paper.

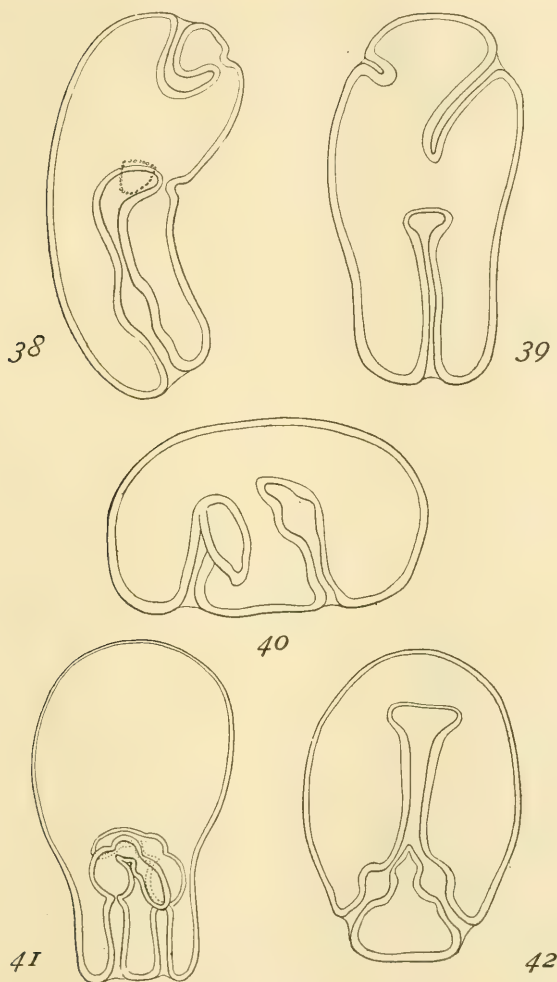
One type of fused twin must, however, be noted here, since there is no regulation back to a single condition. In a considerable number of cases, which probably started out like the parthenogenetic twins shown in figures 25 to 28, fusion occurs at the anterior ends of the equal-sized archentera, and a new type of twin results (figs 33 and 35) in which a common anterior end is produced, but the posterior ends remain separate and open by separate blastopores. Such larvae remain strictly symmetrical and since neither component is secondary, both persist throughout the life of the larva. A still different fate of secondary archentera remains to be noted, in which the blastopore closes and the whole structure first pinches off from the surface, as in figure 36, and subsequently lies as a closed vesicle in the larval body cavity. Sometimes two or more such vesicles are found.

## C. TWINS IN NORMALLY FERTILIZED EGGS

In a considerable number of the control cultures that were run as a check upon the results of parthenogenetic and of hybrid experiments, no particular pains were taken to prevent crowding, and as a consequence some of the larvae encountered unfavorable growth conditions that retarded their development. The experimental practice in the case of these control cultures was to pour off from the surface of each dish the upper third of the sea-water, which contained most of the normal active larvae; for, especially in somewhat crowded cultures, the larvae that are vigorous enough crowd to the surface presumably for oxygen. The larvae thus poured off from the surface are provided with fresh sea-water and are therefore enabled to recover from any temporary inhibiting influences under which they may have been living. The original dish was refilled with fresh sea-water and kept for some time for purposes of comparison. It was found that a very large proportion of the larvae left after pouring off the surface were subnormal in various respects. On the bottom of the dish, apparently unable to rise from their position, were numerous examples of exactly the kind of abnormal larvae, including the various types of twins, that were found in parthenogenetic strains. It therefore seems to be a safe conclusion that these larvae are in fact parthenogenetic. Apparently these eggs are in a physiological state in which they are incapable of fertilization, but are capable of cleavage without membrane formation.

It is among the larvae that rise to the surface and are put into fresh sea-water that one finds occasional instances of more advanced types of twins or double monsters than are found either in parthenogenetic or in hybrid strains. Several types of these twins will now be described in detail.

*a.* One very interesting type of elongated larva was found (fig. 38) in which two well-developed archentera appeared, one in the normal position, the other, somewhat smaller, at the opposite end. Both archentera had widely open blastopores, both were curved toward the ventral side and were sending out the endodermal evagination for a mouth. In addition both had



(Figs. 38 to 46 are from normally fertilized but crowded cultures)

Fig. 38 A very advanced twin larva in which much differentiation has taken place (see text).

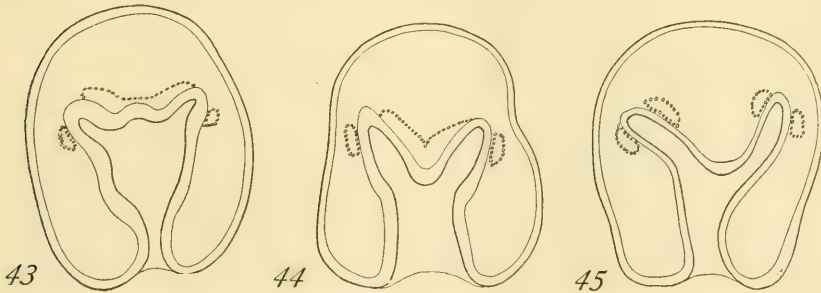
Fig. 39 A somewhat less advanced triplet larva.

Figs. 40, 41, and 42 Examples of advanced twin larvae of various types showing differentiation of oesophagus, stomach, and intestine.



fairly deep invaginations of the ectoderm destined for the stomodaeum. One might expect such a larva as this to display evidences, in connection with its locomotion, of discord between the two components, but this was not the case, for the larva went ahead at a rapid rate revolving on the long axis and always keeping that end in advance at which the smaller, evidently secondary, archenteron opened, so that this second individual proceeded backward with its blastopore ahead and its own head end behind. This twin larva was kept under observation for over a week and little further advance was noted. No distinct ciliated bands were formed, but small vesicles of mesenchyme about the anterior end of the posterior archenteron (lacking in the anterior archenteron) indicated an attempt to form the hydro-enterocoel cavities. The presence of the secondary archenteron evidently inhibited the full consummation of this process. Another unusually large, active larva of somewhat similar type, but with two secondary archentera, is shown in figure 39. In this case no hydro-enterocoel pouches were produced, although in normal larvae of the same age and size an advanced stage of coelom differentiation had been reached. The various types of twin larvae live for a long time. In figures 40, 41, and 42 are shown three larvae drawn at twenty, twenty-four, and eighteen days, respectively. They all show considerable differentiation of the alimentary tract into oesophagus, stomach, and intestine. Figure 40 probably arose from a larva similar to that shown in figure 12. The left-hand archenteron has undergone the flexure of the stomach characteristic of normal larvae. The right-hand archenteron, however, is somewhat less advanced. The larva shown in figure 41 presents an intricate appearance. The two archentera show advanced differentiation and are mirror-image duplicates so far as differentiation is concerned, both showing the same flexures and the same degree of differentiation of the oesophagus, stomach, and intestine. Figure 42 shows a rare case of an advanced larva of the type shown in figure 33. Here the anterior, fused region is relatively inhibited, while the stomach and intestine are clearly differentiated.

b. An entirely new type of twin larva was noted in two independent cultures of normally fertilized eggs. On the fourth or fifth day after fertilization it is noted that many larvae exhibit a forking of the anterior end of the archenteron. This forking is sometimes very incomplete, the anterior end of the archenteron merely flaring out and becoming hammer-headed. Many stages of forking (figs. 43, 44, 45) far more complete than this occur, however, and in the more extreme cases it becomes perfectly obvious that the archenteron has undergone fission and that two distinct anterior ends result. In several cases each of these 'heads' formed paired hydro-enterocoel pouches, both right and left pouches being distinct on both components (fig. 45). In

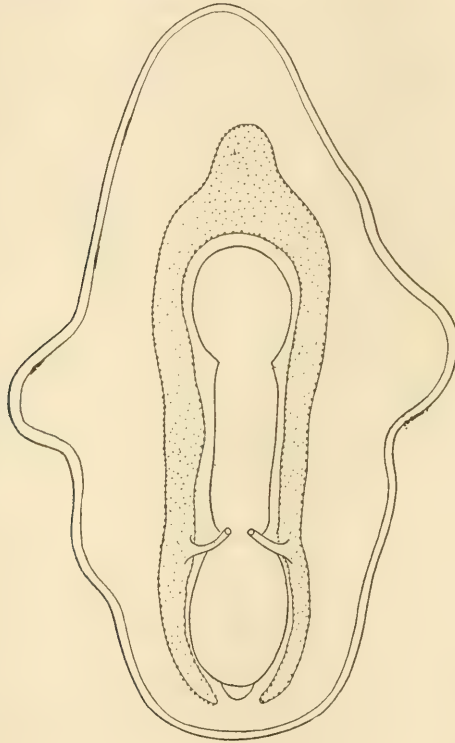


Figs. 43, 44, and 45 Examples of the so-called 'dicephalous' larvae

other cases the outside pouches were distinct, but the inside ones were incompletely separated (fig. 44) or entirely fused into a single median pouch (fig. 43). Out of a large number of these 'dicephalous' larvae only a very few lived beyond the sixth day, and in no case was there any considerable progressive differentiation beyond that just described.

c. A third type of anomalous larva about which there is still some question, but which I believe belongs to the same series and is due to the same type of cause as the twinning types just mentioned, is the advanced bipinnaria larva with paired madreporic pores and pore canals. In one apparently quite healthy culture of larvae from normally fertilized eggs of *Patiria* were found twenty-seven anomalous specimens in which there were

paired madreporic pores, or at least paired pore canals. Figure 46 is a simplified drawing of one of these. As is well known, the advanced bipinnariae of asteroides normally develop madreporic pores and pore canals only on the left side, and this asymmetrical



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Fig. 46 Outline of advanced bipinnaria larva seen from the ventral side, to show especially the coelomic system and the paired madreporic pores and pore canals. Twenty-six other larvae of this kind were found in one culture.

growth initiates the change from bilateral to radial symmetry. The production of the paired condition in many larvae, instead of the single condition typical for the group, is, in our opinion, to be interpreted as only another phase of twinning. These larvae are looked upon as the result of a partial physiological isolation of the bilateral halves of the larva, the consequence of

which is that each side acts independently of the other and produces all of the structures characteristic of the species. Neither side is in these circumstances a subordinate or inhibited side, but both sides are of equal dominance. From such larvae as these there arise, without question, the occasionally observed adults with double madreporic plates and stone canals. A more detailed account of these double-pored larvae is given in a separate paper (Newman, '20 b).

*Summary of data on twins in Patiria*

1. For present purposes all forms of larvae are classed as twins in which doubling of normally single structures occurs; for twinning is believed to be, in last analysis, a process involving duplication of originally single structures.

2 Several distinct types of twinning are found in *Patiria* larvae, of which the following is a list:

a. Completely separated half- and quarter-sized blastulae, derived through physiological isolation of the blastomeres of the two-cell or of the four-cell stages.

b. Partially separate blastulae, double monsters, derived through incomplete physiological isolation of the blastomeres of the two-cell stage.

c. Double-monster gastrulae derived by gastrulation of forms described in b.

d. Gastrulae with two, three, or more archentera, derived from blastulae in which the axial gradient or general polarity had been either eliminated entirely or had been radically disturbed.

e. Advanced gastrulae or bipennariae in which the distal parts of the paired archentera have undergone fusion and in which the proximal parts of the archentera and the blastopores remain separate.

f. Advanced gastrulae or bipennariae in which the paired archentera remain separate throughout life, involving the differentiation of two sets of alimentary derivatives.



*g.* Middle and advanced gastrulae and early bipennariae in which the originally single archenteron undergoes twinning at its free or distal end, resulting in 'two-headed' forms in which the two forks of the archenteron both give off hydro-enterocoel pouches.

*h.* Advanced bipennariae in which the madreporic pore and pore canal, normally confined to the left side, is found in a fully paired condition, the structures on the right being often quite as well developed as on the left.

These twin embryos and larvae are believed to represent a logical series and to be the result of the same or similar causes, though they are not all found under the same conditions. The diversity of types is believed to be due to differences in the time of onset of the causal factors and to the varying degrees of severity of the inhibiting agents.

3. Twins are found under three different conditions: *a*) as the result of spontaneous parthenogenesis; *b*) as the result of hybridization; *c*) as the result of overcrowding of normally fertilized eggs.

The largest variety of twins is found in the third class, i.e., from eggs normally fertilized, because there are always parthenogenetic eggs in such cultures, and because, in addition to all of the forms found in hybrid strains, some of the more advanced strains occur only in normally fertilized cultures. Although the greatest variety of twins is found in normally fertilized cultures, the largest percentage of twins occurs in hybrid strains, in some cases about 50 per cent of all living larvae being twins of some sort. Parthenogenetic twins as a rule die early, and in no case do they reach a true bipinnaria condition. Hybrid twins are distinctly more viable and develop further. Twins in normally fertilized cultures show the greatest viability and reach the most advanced conditions.

4. A very large percentage of the twins with two or more archentera undergo regulation and become more or less normal-appearing single larvae. This phenomenon has been studied in detail and is to be brought out subsequently in a separate contribution.

5. The one factor in common among the three methods of producing twins in *Patiria* has to do with a more or less pronounced retardation of the developmental rate, accompanied by a decrease in metabolic intensity. In the case of parthenogenetic eggs, cleavage begins at least three hours later than in normally fertilized eggs. The retardation here is extremely early and the results are seen in the physiological isolation of the blastomeres of the two- and four-cell stages, and the formation of completely separate twin blastulae. In the case of hybrid larvae, retardation is neither so early nor so severe in its onset as in parthenogenetic forms, nor so prolonged in its action. The consequence is that the results are not clearly noticeable until the onset of gastrulation. Excluding from consideration the parthenogenetic eggs that occur in normally fertilized cultures the retardation of development in larvae resulting from normally fertilized eggs is not marked during the cleavage period, but becomes evident when the larvae hatch and swim to the surface. There are no twins among those that hatch first and are earliest drawn off from the surface. Among the larvae that hatch later, however, there are twin larvae of several types, some simulating those in hybrid cultures, others, however, such as the 'double-headed' and 'double-pored' types, have no parallels in hybrid cultures. These larvae show effects of retardation at later stages than do twins produced under either of the other conditions previously dealt with.

*One conclusion, therefore, seems to be evident, that, in Patiria at least, twinning of all sorts is intimately associated with retarded development.*

#### DISCUSSION AS TO THE CAUSES OF TWINNING

The writer has for some years entertained a physiological theory as to the causes of twinning, derived from his studies of the striking case of twinning (specific polyembryony) in the nine-banded armadillo. This theory was expressed most recently (Newman, '17) in the volume, *The Biology of Twins*, though it had been stated some years earlier (Newman, '13). The situation in the armadillo is as follows. The egg is at first single, as

in other mammals, and remains single while it descends the oviduct and even after it reaches the uterus. When the egg reaches the uterus it has undergone 'embryonic germ-layer inversion,' so that the ectodermic vesicle is at least partially surrounded by endoderm. Patterson ('16) has made the very significant observation that the embryo stops developing at an early gastrula stage and remains quiescent for several weeks. Toward the close of October, after having been at a developmental standstill for a long time, floating free in the uterus without any nutritive or respiratory connection, a significant event takes place: the embryo accomplishes *placentation*. Development is resumed when the egg undergoes the process of primary placentation in October and new growth vigor is derived from the nutritive supply thus obtained. The period of retardation has, however, been so prolonged that the embryonic axis has been largely obliterated and, when rejuvenation or recovery takes place, one location in the ectodermic vesicle is as likely to become the apical point of the new axis as another. As a matter of fact, two points, favorably situated with reference to the conformation of the uterus, assume the initiative and become two new head ends or apical points. A little later two other points, not so favorable situated, become similarly physiologically isolated and form two secondary head ends. The result is the formation of one primary and one secondary individual out of each lateral half of the ectodermic vesicle. The four apical points grow as though each were a whole independent embryo and each forms its own amnion and its entirely separate placenta, though they remain enwrapped in a common birth robe or chorion. The phenomenon is one involving physiological isolation, through retardation, of parts of the ectodermic vesicle, that part of the embryo that takes the initiative in development. The succeeding processes of complete separation of the four quadruplets are the normal sequelae of the above-described initial steps and need not concern us here.

Do the results described above for the starfish *Patiria* tend to strengthen this theory as to the causes of twinning? I believe they go far in that direction. Each of the three methods of



producing twins involves retardation, physiological isolation of parts of the egg or embryo, the origin of separate growing points, or apical points, and the consequent doubling of normally single embryos or structures. What, in *Patiria*, we may ask, corresponds with the renewal of developmental vigor supposed to be the result of primary placentation in the retarded armadillo embryo? In every case where good results were obtained and twins developed beyond an early gastrula stage, these results were due to separating the living embryos from dead eggs and placing them in fresh sea-water. The vessels containing twin larvae were relatively roomy for the number of larvae present, and water was changed at regular intervals during the periods of observation. Doubtless, then, the rejuvenation of the retarded embryo, which permitted a continuation of development in otherwise doomed eggs and embryos, resulted from suddenly improved environmental conditions. Many larvae were short-lived, showing an inability to recover from the lethal changes that had set in, others recovered to become very abnormal single embryos, but a large number of twin larvae were nearly always present whenever eggs that had been retarded were reinvigorated by improved conditions. It is probable, also, that recovery in the case of hybrids, and possibly in other cases, was not strictly due to improved external conditions, but was a matter of internal adjustment or of acclimation. It is hardly possible from our data to decide between these alternatives in any single case. Physiologically speaking, however, acclimation and recovery are so closely interrelated that it seems probable that they are in most cases much the same process, or at least phases of one process which for want of a better word may be called rejuvenescence. What appears to happen in all cases of twinning is a primary dedifferentiation of the original apical point and a subsequent redifferentiation of two or more apical points in the place of one. There are numerous evidences of dedifferentiation, partial or complete, in the retarded embryos and larvae of *Patiria*, and there are equally numerous instances of redifferentiation of plural apical points in an embryo formerly with a single apical point. The simplest expression of this process



is seen in the case of the much-retarded parthenogenetic larvae of *Patiria*, where blastulae, at first quite devoid of axes of polarity and with no pronounced apical points, undergo gastrulation at points that are relatively basal; for it is at the basal end of normal larvae that invagination of endoderm takes place. In such larvae as that shown in figure 13 the whole periphery is physiologically basal, and gastrulation occurs anywhere or everywhere, and as many gastrulations occur as there is room for on the surface.

It is further to be noted that retardation has its effects, whether expressed in twinning or in other ways, only at such stages and in such places when and where some critical change, involving a process of unusual delicacy, is taking place, and where even a slight or temporary retardation would result in a sufficient developmental let-down to bring about physiological isolation of tissues to the right and left of the original apical point.

*The probable causes of twinning in other species*

One of the classic studies of experimental twinning is that of O. Schultze ('94), using the eggs of the frog. He discovered that if he turned the egg upside down when in the two-cell condition there frequently developed twin or double-monster larvae and that the axes of the twin embryos bore no constant relationship to each other. Sometimes the anterior ends pointed in exactly apposite direction; sometimes they were at right angles to each other, and sometimes they bore a mirror-image relation to each other. It is obvious from Schultze's account that inversion of the frog's egg decidedly retards development and necessitates a more or less complete dedifferentiation of the blastomeres, followed by a redifferentiation of a new polarity, involving the formation of a new apical point in each blastomere. If the dedifferentiation were sufficiently thoroughgoing, there resulted complete physiological isolation of the two blastomeres, and separate twins were formed. If dedifferentiation were less complete, double monsters or conjoined twins were produced. The essential fact is not that the eggs were inverted and that specific gravity caused a restratification of formative materials,

but that the developmental momentum was so seriously slowed down that dedifferentiation of the original symmetry relations occurred, and, when recovery came, the unity of the organization had been lost, giving an opportunity for the redifferentiation of two independent apical points and the consequent twin development.

Wilson ('11), in his classic book on the cell, describes and figures various phases of twinning in *Amphioxus*, which remind one strongly of the conditions described for *Patiria*. Not only were dwarf larvae produced, but gastrulae with paired archentera occurred not infrequently. Some of the earlier stages of twinning are seen in which it is obvious that, as in *Patiria*, the blastomeres of the two-cell stage had become physiologically isolated, each following its own cleavage plan, and destined to form double blastulae or gastrulae, either isolated or conjoined. The experimental procedure was the familiar one of shaking the eggs when in the two-cell or the four-cell stages. When the shaking was sufficiently violent blastomeres were physically isolated and produced separate dwarf larvae; but if the shaking was less severe the blastomeres were only physiologically isolated to varying degrees and various types of double monsters resulted. Wilson does not say definitely that the shaking was followed by retardation, but the inference is that the shaking caused a disorganization of the bilateral symmetry relations existing between the blastomeres of the two-cell stage, and the consequent redifferentiation of two apical point and two separate axes.

An interesting case of twinning in plants was brought to the writer's attention last June. In the Santa Clara Valley of California many peach trees bore considerable percentages of twin peaches. These consisted of various stages of double fruits, some almost entirely separate, others merely constricted as to the fleshy parts. On inquiry, it was learned that at the flowering season a severe cold spell had occurred in this region, and this was held to be responsible for the twinning in the fruits. How could this be explained? A theory might at least be postulated to clarify the situation. The unusual cold probably

stopped development in some of the more susceptible trees, and those flower buds that were in the most sensitive condition were most severely inhibited. When seasonable weather returned the flower buds capable of recovery resumed growth, but the most apical region had become so seriously injured that it was incapable of continuing. In consequence, symmetrical regions of the growing tip near the original apical point became the new apical points of the twin fruits.

*The causes of human twinning*

Davenport has recently published data that tend to show that monozygotic twinning is inherited strongly through the male parent. If this finding is valid, how could this fact be shown to accord with the general theory of twinning herewith expounded? If in human beings twinning be a result of temporary retardation followed by recovery, how could the sperm be responsible for retardation? It is evidently true that in human beings, as in other animals, there are varying degrees of compatibility between the eggs of some females and the sperms of some males. Doubtless the eggs of some females are totally incapable of fertilization by the sperms of some males, while quite fertile to the sperm of others. Doubtless also there are many borderline cases that involve relative incompatibility and consequent disharmony and retardation. If retardation be sufficiently severe, physiological isolation might occur at a relatively early period, which would likely result in completely separate twins; but if physiological isolation occurred relatively later, there would be a less complete separation of the twin bodies, and the resultant conjoined twins, cases of dicephaly, spina bifida, and others types of teratological duplication common in human fetuses. Thus twinning might be inherited through the male line owing to some peculiarity of sperm in a given race that has a retarding effect upon the egg.



*Reduplication of limbs a special case of twinning*

In case only certain parts of individuals are reduplicated, it is to be borne in mind that many organs or systems possess separate axes or gradients of their own. The limbs of vertebrates, for example, have their own gradients, with the apical point at the distal end and the basal point at the proximal or attached end. It is not uncommon for limbs to show reduplication, especially under experimental conditions. A very suggestive series of experiments involving the reduplication of limbs has recently been published by Detwiler ('20). He transplanted the anterior limb buds of *Amblystoma* embryos varying distances back from their normal position. These transplants differentiated in their abnormal positions with varying degrees of success, depending upon their distance from the normal locus. Many limbs developed as mirror-image duplicate appendages. According to the author, "there occurred a gradual increase in the number of reduplications as the limbs became transplanted farther and farther away from the normal situation." It is also clearly shown in the author's description that the rate of development becomes progressively slower as the reduplications become more frequent; at least one may infer this from his statements as to the time of appearance of the first reflexes, which appear in about fourteen days in the normal limb; about two days later when the limb is placed one or two somites back; from two to ten days later than normal when placed three somites back; from fifteen to twenty-six days later normal when placed four somites back; from seventeen to twenty-two or more days later when placed five somites back. It would appear, then, that reduplication or twinning of limbs is associated with retarded development. What causes retardation to be more pronounced the farther from the normal position the transplant is placed is another problem.

The writer is confident that other cases of reduplication in development will be found to be associated with retarded development and that the theory of twinning herewith proposed will be found to have a very far-reaching application to all phenomena of twinning in the broad sense of this term.



*In conclusion, then, we may give as our general causal theory of twinning (including the doubling of all normally single structures) that the first step involves retarded development, followed by loss of organization or dedifferentiation; that recovery or acclimation results in the formation of new apical points, and the new apical points form the head ends of new individuals.*

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Resumen por el autor, John F. Fulton, Jr.  
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Sobre la vitalidad de *Actinia bermudensis*. Un estudio sobre la simbiosis.

El trabajo experimental publicado en la presente contribución fué llevado a cabo con el fin de determinar si la asociación entre *Actinia bermudensis* y las *Zooxanthellae* que contiene es una verdadera simbiosis. *A. bermudensis* presenta una notable resistencia a las condiciones desfavorables del medio ambiente; el autor sospechó a causa de esto, que la notable vitalidad de esta actinia pudiera ser debida a la presencia de los organismos con clorofila contenidos en sus tejidos.

En un recipiente cerrado que contenga 100 cc. de agua de mar, la actinia puede vivir durante seis días, en el oxígeno vive siete días y en el aire el mismo tiempo. Cuando se coloca el recipiente en la oscuridad (en cuyas condiciones las algas simbióticas no funcionan) la anémone puede vivir el mismo tiempo. Esto indica que las *Zooxanthellae* no son simbiosiontes verdaderos, y el autor llega a la conclusión de que lo mismo que en el caso de las células clorofílicas de *Convoluta roscoffensis*, la asociación entre los organismos que contienen clorofila y *A. bermudensis* es una relación de parasitismo obligado, viviendo la actinia parásita sobre las *Zooxanthellae*.

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## CONCERNING THE VITALITY OF ACTINIA BERMU- DENSIS: A STUDY IN SYMBIOSIS<sup>1</sup>

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While investigating the pigmentation of several species of actinians common in the Bermuda Islands, the writer observed that *Actinia bermudensis* Verrill possessed great resistance to unfavorable environmental conditions. It had been noted previously that the gastrovascular fluid of this actinian teemed with Zooxanthellae—holophytic flagellates of the suborder Cryptomonadina—and consequently the question arose as to whether or not these organisms increased in any way the vitality of the anemone. The results of the investigation are recorded in the present paper.

The work was carried on during the summer of 1920 at the Bermuda Biological Station for Research, and the writer wishes to express his warmest thanks to Dr. E. L. Mark, to whom he is indebted both for the facilities of the laboratory and for revision of the manuscript.

*Actinia bermudensis* is a deep-red anemone, 20 to 30 mm. in diameter and of slightly greater length, which is found hanging on shaded areas of rock between the levels of high and low tide, and consequently is out of the water at least half of the time. The specimens used in the present study were collected at low tide, when they are found with contracted tentacles hanging limp from crevices in the rock. They were usually obtained by chipping off pieces of the sandstone to which they were attached.

The presence of Zooxanthellae in *A. bermudensis* may very easily be demonstrated by withdrawing a small quantity of the gastrovascular fluid with an injecting needle and examining it on

<sup>1</sup> Contributions from the Bermuda Biological Station for Research, no. 127.



a slide.<sup>2</sup> When a fresh smear of the gastrovascular fluid is observed, the Zooxanthellae are usually in a state of active vibration. Their flagella, though not visible in the living condition, may be observed after adding a drop of a mixture of acetic and osmic acids. The organism contains a yellow pigment, hence the name 'yellow cells' (Geddes, '82), which functions photosynthetically, and it is therefore of interest to learn whether or not the Zooxanthellae present in *A. bermudensis* are truly symbiotic,<sup>3</sup> and whether they are responsible in any way for the great vitality which the anemone possesses. In an effort to answer these questions, a series of simple experiments was made.

These consisted in subjecting specimens of the anemone to varied environmental conditions, and using for controls specimens kept in the dark, but otherwise under the same environmental conditions. Since the Zooxanthellae could be of functional value to the anemone only through action of sunlight, the possible aid derivable from their presence would be cut off so long as the anemone was kept in darkness. The individual anemones kept in sunlight ought, therefore, to exhibit greater vitality than those kept in darkness, if the Zooxanthellae contribute through their photosynthesis to the nutrition of the anemones.

*Experiment 1—Sea-water.* Each of the anemones<sup>4</sup> of the first group was placed in a bottle containing 100 cc. of unsterilized sea-water on July 14th, sealed, and allowed to remain until dead.<sup>5</sup> On July 19th all of the actinians appeared to be alive.

<sup>2</sup> Zooxanthellae are common in most actinians. The writer has noted them in *Condylactis passiflora* D. & M. (in which they are responsible for the color of the tentacles), *Aiptasia tagetes* D. & M., and *Epicystis osculifera* Verrill; Cary ('11) has reported them also in: *Aiptasia pallida* Ag., *A. annulata* Andres, and *Cylista leucolena* Ag.; they have also been described by Heilprin ('89) for many species of Zoanthidae. In the course of the present study, Zooxanthellae were also noted in several species of gorgonians, particularly in *Plexaura flexuosa* Lamx. In the rose coral, *Isophyllia* (dipsacea and fragilis), is found a holophytic organism which is probably closely allied to Zooxanthella.

<sup>3</sup> Winter ('07) has shown that Zooxanthellae are symbiotic in *Peneroplis* (a foraminiferon).

<sup>4</sup> Three animals were used for each experiment.

<sup>5</sup> The only way by which it could be determined with absolute certainty whether or not an animal was dead was to remove it from the air-tight bottle

On the 20th the water in each of the bottles had become cloudy, giving evidence of putrefaction. All three of the animals were, therefore, removed to fresh sea-water; two were dead, but one revived shortly after it was placed in running water. This means that a specimen of *A. bermudensis* is able to live sealed in 100 cc. of unsterilized sea-water for from five to six days. Sterilized sea-water was also used, but the results did not differ. In this connection it is interesting to note that of several other animals (a star-fish, a sea-urchin, a coral—*Isophyllia*—and an ascidian) which were treated in the same way none lived more than twenty-four hours, and most of them less than twelve.

The animals which were placed under corresponding conditions, but in the dark, all proved to be alive at the end of the fourth day, and in another series of experiments one lived for five days in the absence of light. This experiment, therefore, would seem to cast doubt upon the assumption that the *Zooxanthellae* are true symbionts.

*Experiment 2—Air (unsealed).* On July 14th three *A. bermudensis*, still attached to the rocks occupied by them when collected, were allowed to remain in the air (but not in direct sunlight) wholly out of contact with sea-water. As this species is usually found above low-tide level, it could reasonably be expected that the animals would survive for a considerable length of time under the conditions of the experiment. No effort was made to regulate the temperature, since in their natural habitat there is a moderately great variation in this factor. In this experiment the anemones were not exposed to direct sunlight, inasmuch as they usually frequent shady crevices.

After six days in the air the three specimens gave the appearance of large dried raisins attached to the rocks. Believing that the animals must certainly be dead, two of them were placed in sea-water; in less than five minutes they had revived, with tentacles expanded, and appeared normal in every respect. On

and place it in running sea-water. Consequently, in cases in which the animal revived when removed from its confinement, it was impossible to know how long it would have lived under the adverse conditions. The results, however, are sufficiently striking to bear evidence of the great vitality which the animal possesses.

the eighth day the third specimen, which had shriveled to about one-tenth its normal size, was placed in water, and, though more slowly, it too revived.<sup>6</sup> Several other specimens were placed in direct sunlight (out of contact with water); they lived but four days. It would seem, therefore, that death occurs as soon as the water in the gastrovascular fluid has disappeared. This observation brings up many interesting problems which would well repay further investigation. In the first place, an anemone out of contact with the water cannot obtain food. How long, then, can an actinian live without food? Does the anemone ingest the Zooxanthellae themselves, as Keeble and Gamble ('07) have shown to be the case with the green cells in the turbellarian worm *Convoluta roscoffensis*? Since it is known that the internal fluids of nearly all of the marine invertebrates are isotonic with the sea-water in which they live (Fredericq, '85), what occurs when the gastrovascular fluid of a sea-anemone evaporates; does the saline concentration increase, as one might expect, or does the organism possess some compensatory mechanism for preventing such an increase?

The control specimens, which were placed in the dark room, survived a period of six days without water, and at the end of that period showed a degree of vitality which was as great as, if not greater than, that of the corresponding ones in the light.

This, again, throws doubt upon the symbiotic character of the organisms which are harbored in the gastrovascular fluid, and it also gives fair indication that life continues so long as there is sufficient water present to carry on the metabolic activities.

From the two experiments just recorded it was at once evident that *A. bermudensis* is capable of existing upon a remarkably small amount of oxygen, since the animal remained alive for six days upon the quantity of oxygen which is normally dissolved in 100 cc. of water.<sup>7</sup> It was therefore of interest to see how long an animal could exist in the complete absence of oxygen.

<sup>6</sup> It died, however, the following day.

<sup>7</sup> In this connection it may be noted that, according to Vernon ('96), the respiratory exchange is lower for coelenterates than for any other group of animals, save possibly certain ascidians. For further details see Krogh's ('16, p. 144) monograph on the respiratory exchange of animals.



*Experiment 3—CO<sub>2</sub>.* Accordingly specimens were put in 100 cc. of carbon dioxide contained in an inverted large-mouth bottle, which was placed in a small finger-bowl with sufficient sea-water to seal the edges. An atmosphere of carbon dioxide not only deprives the animal of oxygen, but is at the same time toxic to the organism. Moreover, if the anemone should prove better able to live in such an atmosphere when exposed to sunlight than when put in the dark room it would establish definitely the symbiotic<sup>8</sup> nature of the Zooxanthellae which it contains. Consequently, the experiment was repeated several times with fresh animals to insure accuracy.

When *A. bermudensis* is placed in carbon dioxide the animal at once becomes limp, and fails even to contract its tentacles, thus giving the appearance of being completely overcome. Gradually, however, after several hours, it commences to recover. The body slowly regains its tone and (at the end of six hours) the tentacles are slowly contracted. Twenty-four hours after being placed in CO<sub>2</sub>, all of the animals appeared quite normal. At the end of forty hours one specimen had commenced to slough off a reddish mucus, which is always indicative of approaching death. After a lapse of forty-eight hours all of the specimens were dead. In a repetition of this experiment one actinian lived in carbon dioxide until the third day.

The control specimens, which were placed in the dark room, showed practically the same degree of vitality as those which had remained in the sunlight. After one day in the CO<sub>2</sub> they were still alive, and did not die until after from forty to forty-five hours' confinement. As with the two preceding experiments, this one also gives a very real indication that the Zooxanthellae are not symbiotic in *A. bermudensis*.

*Experiment 4—Oxygen.* Three specimens of *A. bermudensis* were put into an atmosphere of pure oxygen, the container being arranged as in the preceding experiment. Under these circumstances, two individuals died on the seventh day and one revived when put into sea-water on the eighth day. The

<sup>8</sup> When 'symbiotic' is used with reference to the Zooxanthellae, it refers only to a state of photosynthetic symbiosis.



vitality of the control specimens was slightly less; two died after six days and one on the seventh.

*Experiment 5—Air and moisture.* Each of three specimens was sealed in 100 cc. of air, as in the two preceding experiments. The fact that in these experiments the bottles were sealed with water prevented the excessive evaporation and consequent dryness which resulted in experiment 2. In that experiment the greatest longevity was eight days. The present experiment was commenced on July 15th at 9:00 A.M., and at 12 M., July 26th (when

TABLE 1

*Showing length of life of Actinia bermudensis under various environmental conditions*

	EXPERIMENT				
	No. 1	No. 2	No. 3	No. 4	No. 5
Actinia bermudensis in.....	Sea-water 100 cc. (sealed)	Air (un- sealed)	CO <sub>2</sub> 100 cc. (sealed)	O <sub>2</sub> 100 cc. (sealed)	Air 100 cc. (sealed)
Sunlight.....	6 days	7 days	45-48 hours	7 days	11 days (exp. dis- con- tinued)
Darkness.....	5 days	6 days	40-45 hours	7 days	7 days (exp. dis- con- tinued)

the experiment had to be discontinued), all three of the actinians were still alive and appeared normal in every respect, having lived in a sealed bottle for eleven days.

The control experiment was discontinued at the end of the seventh day; there was no indication, however, of decreased vitality as a result of the darkness. Table 1 gives in brief the results of the experiments just described.

What deductions can be made from these experiments? Let us examine first the conclusions which have been reached by previous investigators.

The presence of chlorophyll in animal tissues has been repeatedly demonstrated. Among the Protozoa its occurrence is a matter of common knowledge (Sallitt, '84; Lankester, '85). By means of the spectroscope Lankester as early as 1868 had made it clear that chlorophyll was present in at least one sponge (*Spongilla fluviatilis*); later, the investigations of Sorby ('75), MacMunn ('88), and Krukenberg ('84) established the presence of chlorophyll in seventeen other species of sponge. In the coelenterates chlorophyll is not to be found as a separate animal pigment, but as chromatophores of intruding algal and protozoan cells. A similar condition exists among certain of the marine Turbellaria. The question as to whether or not these intruding algal cells are photosynthetically symbiotic has excited great interest and not a little dispute; it is a question, moreover, which, as far as the coelenterates are concerned, has never been satisfactorily settled.

The presence of 'yellow cells' in the body of sea-anemones and of radiolarians has long been known. Johannes Müller held at first that they were concerned in the reproduction of the Radiolaria, while Haeckel ('62, p. 136) assigned a nutritional function to them, comparing them to wandering liver cells. Cienkowski ('71) was the first to look upon these organisms as parasitic algae. He showed that they not only could survive the death of the anemone, but could also live and reproduce outside the animal body. Richard Hertwig ('76) refused at first to accept Cienkowski's conclusion that the 'yellow bodies' were parasitic, believing that they were a part of the animal tissue; later, however, the brothers Hertwig ('79), and still later O. Hertwig ('83), abandoned this view on confirming Cienkowski's observation that the organisms were capable of an independent existence. This observation was further confirmed by Brandt ('81 b). Geddes ('82) proved that the 'yellow cells' from *Anthea cereus* give off oxygen in the presence of sunlight; however, he was unwilling to admit (as was also Lankester, '82) that the cells were parasitic; but held, with Richard Hertwig, that the animal was capable of manufacturing its own chlorophyll. This view was vigorously opposed by Brandt ('81 a), who was so thoroughly convinced of the parasitic nature of these forms that he established the genus

Zooxanthella and described the species from *Collozoum inerme* as *Z. nutricula*. He confirmed and extended his observations in later papers (Brandt, '82, '83). In the latter paper ('83) he observed that if *Sagartia* or *Aiptasia* are put in the dark for three days, they extrude all of their yellow cells, and if subsequently put into filtered sea-water they continue to live uninfected by the Zooxanthellae. Though Keeble and Gamble ('07, p. 171) cast doubt upon the accuracy of Brandt's experiments, the writer has himself observed that *Condylactis passiflora* lost its yellow cells after two days in darkness (as indicated by the loss of color of the tentacles), and that three days were required for reinfection, even in unfiltered sea-water. Moreover, many authors (Beyerinck, '90; Famintzin, '89, '91; Dantec, '92; Dangeard, '00, and Keeble and Gamble, '07) have reported having made cultures of parasitic algae taken from protozoans, actinians, and turbellarians. The authors last mentioned (Keeble and Gamble) have made a thorough-going investigation of the green and yellow cells of the turbellarian worms *Convoluta roscoffensis* and *C. paradoxa*, and their results demand very careful consideration (Gamble and Keeble, '03; Keeble and Gamble, '05, '07; Keeble, '08, '10).

*Convoluta roscoffensis* is a slender green worm peculiar to the coasts of Brittany. It occurs in 'spinach-green' patches, and has as a distinguishing feature of its ecology the habit of sinking at night below the surface of the sand, coming out only during the sunlight. Previous investigators (Geddes, '79 a, '79 b; Haberlandt, '91, and Georgévitch, '99) had concluded that *Convoluta* ingested no solid food of its own, but that is depended entirely upon the nutritive substances supplied by the green algae; they believed, that is, that in *C. roscoffensis* there exists a condition of true photosynthetic symbiosis. Gamble and Keeble, however, have found after many careful experiments that before maturity *Convoluta* 'feeds and feeds voraciously.' In contradiction to the observations of Geddes and of Haberlandt, that the green worm dies after three days in a dark room, they find that *Convoluta* is able to remain in darkness for more than a fortnight. They observe, moreover, that the starch from the green cells disappears only with great slowness during the confinement.

They conclude, therefore, that *C. roscoffensis* has not lost its power of independent nutrition, and that it obtains but little food from the green cells. They find, in addition, that the green cells—Zoochlorellae<sup>9</sup>—are not developed by the animal itself, but are organisms which infect the animal from without, entering the host usually as colorless leucoplasts, which only subsequently develop their green pigment. As to the physiological relation of the algae to the worm, Gamble and Keeble conclude that it changes with development, passing from one of true symbiosis, in the early stages of development, to one in which *the turbellarian is parasitic upon the algal cells*. The evidence for this last conclusion was threefold: 1) in the first place, during starvation *Convoluta* digests its green cells; 2) the Zoochlorellae never reproduce after infecting the worm; 3) the association of the green algae with the turbellarian is followed by a degeneration of the worm's excretory system—the algae utilizing the worm's nitrogenous waste, thus functioning as an excretory apparatus for the animal. It must be emphasized, therefore, that the association between the algal cells and *C. roscoffensis* is not one of symbiosis, but a relation of obligate parasitism.

Let us return now to the condition in *Actinia bermudensis*.

The results for *A. bermudensis* recorded in the first part of the present paper (table 1, p. 358) seem to agree in many ways with those of Gamble and Keeble for *C. roscoffensis*. In the first place, there is no evidence that the products of photosynthesis from the Zooxanthellae assist in the nutrition of the actinian. Furthermore, it is probable, from certain observations of Brandt ('83) and from those which the writer has made upon *Condylactis passiflora*, that during starvation the anemone feeds upon the 'yellow cells' rather than upon their photosynthetic products. It seems a safe deduction, also, that, as in *Convoluta*, the 'yellow

<sup>9</sup> It is largely a matter of personal judgment whether one considers Zoochlorellae and Zooxanthellae as Algae or Protozoa. Minchin ('12) has adopted the arbitrary method of considering all Zoochlorellae—forms having green pigment—as Algae, while the Zooxanthellae—with yellow pigment—he classifies as Protozoa. This classification, being convenient, has been adopted in the present paper.



cells' of *A. bermudensis* facilitate excretion by utilizing the nitrogenous waste, as well as the carbon dioxide, from the animal. In other words, the condition of association would seem to be one in which the actinian is parasitic upon the *Zooxanthellae* which it contains.

Arndt ('13) has recently published certain results which are not entirely in agreement with my own conclusions. He has shown that the fat globules in the entoderm and ectoderm of the anemone *Heliatis bellis* appear to be identical with the lipoid substances in the *Zooxanthellae*, and concludes that the cells must function in the nutrition of the actinian. He believes that the fat globules are photosynthetic products, and holds, therefore, that the association must be one of true symbiosis. This conclusion does not seem to be entirely justifiable, inasmuch as the evidence for the identity of the fat in the animal tissue on the one hand and that of the *Zooxanthellae* on the other is by no means conclusive.

#### CONCLUSIONS

*Actinia bermudensis* possesses a remarkable resistance to unfavorable environmental conditions (table 1). Sealed in 100 cc. of sea-water it will live for six days; in  $O_2$  for seven days, and in 100 cc. of air (sealed by water) for more than eleven days.

The holophytic organisms (*Zooxanthellae*) do not materially assist the actinian in resisting unfavorable conditions, for when placed in the dark the animal is as well able to resist such conditions as it is in the light.

As with the green cells in *Convoluta roscoffensis*, so well described by Keeble and Gamble, the association between the *Zooxanthellae* and *A. bermudensis* is probably one of obligate parasitism—the actinian being parasitic upon the *Zooxanthellae*.

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Sobre las propiedades fisiológicas de las gonadas como determinantes de los caracteres somáticos y psíquicos.

#### IV. Transplantación de las gonadas en el conejillo de Indias.

Los ovarios injertados en machos jóvenes, previamente castrados, del conejillo de Indias persisten durante un periodo de seis a nueve meses. Después de extirpados y seccionados el aspecto microscópico es todavía muy característico del ovario, existiendo folículos de Graaf. A consecuencia del injerto ovárico el macho posee pezones grandes, bien redondeados, en las glándulas mamarias, las cuales pueden compararse a las de la hembra preñada (modificación somática). No se presenta modificación psíquica, sin embargo. Los animales operados no dieron muestra alguna de inclinación maternal hacia las crías y en algunos casos retuvieron el comportamiento del macho. El tejido testicular implantado debajo de la piel o el peritoneo de las hembras puede encontrarse creciendo con un aspecto sano varios meses después, aun cuando difiere considerablemente del tejido testicular normal.

El estudio microscópico revela que algunos injertos retienen una cantidad considerable del epitelio germinativo de los tubos seminíferos, muchas de cuyas células presentan figuras mitóticas; generalmente, en los injertos de testículo en los mamíferos, solamente persisten las células de Sertoli. En algunos de los injertos la espermatogénesis continúa durante algún tiempo, pero no produce espermatozoides maduros. Las modificaciones somáticas asociadas con el injerto de testículo consisten en cambios de los órganos genitales externos. El clitoris de la hembra experimenta una hipertrofia y produce un resultado final que se asemeja más al macho que a la hembra. La modificación psíquica consiste en cambios de las reacciones sexuales del animal, que reacciona en presencia de otras hembras y machos como un macho típico. No existe indicación alguna sobre la acción antagónica entre las secreciones de las glándulas sexuales adultas del sexo opuesto.

## ON THE PHYSIOLOGICAL PROPERTIES OF THE GONADS AS CONTROLLERS OF SOMATIC AND PSYCHICAL CHARACTERISTICS

### IV. GONAD TRANSPLANTATION IN THE GUINEA-PIG

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FOUR FIGURES

#### INTRODUCTION

In former papers<sup>1</sup> the writer has presented certain observations on the effect of gonad transplantation in young castrated animals of the opposite sex; all observations so far considered refer to the white rat. A similar group of experiments, using the guinea-pig as the experimental animal, were begun before the previous experiments were brought to a close, and a report of these latter experiments is embodied in the present paper. The experiments were conducted in order to study the physiological effect of the internal secretions of the gonads on the somatic and psychical characteristics of the animals.

Since 1910 Steinach has published several papers on his study of the internal secretions of the gonads and their influence upon somatic and psychical differentiation in the rat and guinea-pig. By transplanting gonads from one young animal to one of the opposite sex (rats and guinea-pigs), the latter having its own sex glands removed, he maintains, 1) that a young female becomes masculinized as it grows to an adult and, 2) that a young male in like manner is converted unto a female-like animal. In this transformation the 'masculinized female' grows to a larger size, increasing in weight relative to other spayed females not receiving testicular grafts: the hair coat becomes male-like: the skeleton is different from that of a female: the condition of the fat deposit

<sup>1</sup> See Moore, '19, '20.

is reversed from the normal; the animal is more pugnacious and possesses male instincts, reacting as a male rather than as a female. The 'feminized male' develops into a female-like animal as shown by changed body weight and length (relative), change of hair coat, by the development of the mammary glands (guinea-pigs only), by skeleton changes, by becoming more docile, less pugnacious, and by the acquisition of the female behavior and the development of maternal inclinations toward the young, even suckling the young (guinea-pigs).

Furthermore, Steinach maintains that the secretion of a gonad graft promotes the development of the 'homologous' secondary sex characters of the host and at the same time inhibits the 'heterologous' characters; i.e., an ovary will promote the female characteristics in a developing male animal and inhibit the somatic or psychological characteristics of the male. To him this indicates an antagonistic action between the secretions of the two sex glands of such a nature that not only does the presence of a graft in a castrated animal of the opposite sex inhibit the somatic growth and differentiation and the psychological nature of the animal, but that the presence of one sex gland in an animal will prevent the growth of the opposite gland if attempts are made to transplant the latter gland. By transplanting simultaneously portions of the two opposite sex glands into the same infantile, castrated animal Steinach was able to obtain some persistence of the sex glands for short periods of time, and concluded from this that the antagonistic action between the two glands had been partially overcome.

In former papers ('19) the writer offered criticisms relative to certain criteria employed by Steinach as an indicator of maleness and femaleness. Attention was directed to the facts presented by Stotsenburg ('09, '13) in reference to the effects of the sex glands upon the normal growth of the male and female rats. This investigator showed clearly that in rats the testis had absolutely no effect upon the growth of the animal; the curve of growth for castrated males was almost identical with that for normal unoperated males. In addition, he showed that after the removal of the ovary (complete spaying) the growth curve of the spayed animals increased 17 per cent to 30 per cent above



that of normal females. The increase in weight in female rats, into which a testis has been grafted, is then due only to the removal of the ovary, and not to a secretion from the implanted testis, as Steinach has maintained. The same criticism holds good for body length of the animal. A table of weights compounded from a series of weighings made on eight animals of the same litter,<sup>2</sup> in five of which transplanted gonads of the opposite sex than the host were growing, shows clearly that little if any dependence can be placed on the weight of an animal as an indication of its sexual nature. In the same paper criticisms were offered relative to other indicators of the sexual nature of the animal, but my observations upon the psychical influences of the implanted gonads substantiate those of Steinach to some extent. Some of the female rats in which a part of the testis was successfully grafted behaved in a typical manner. And some of the males in which ovarian grafting was successful exhibited unmistakable maternal behavior toward the young.

It must be emphasized, however, that many pitfalls beset attempts to analyze the sexual nature of either a rat or a guinea-pig by its behavior, and only the most obvious reactions should be used as an indication of the effects of a sex-gland graft.

Sand ('20), working with guinea-pigs, substantiated many of the claims of Steinach, but disagrees with the latter that an antagonism exists between the sex glands. He was successful in implanting an ovary into the substance of a testicle and claims that he could notice momentary changes in the psychical nature of the animal even within the course of a single hour; it is first a female in behavior and immediately afterward is a male. He was not able to obtain growth of a subcutaneous graft of a sex gland if the glands of the host remained intact, and his somewhat elaborate hypothesis explaining this failure is interesting if not essential.

In 1920-21 the writer published the results of many operations in which the portions of the sex gland of the rat were grafted into an animal of the opposite sex, without the removal of the sex

<sup>2</sup> See Moore, '19, page 142 (table).



glands of the latter.<sup>3</sup> An account of twenty-eight successful grafts proved that the two opposite sex glands can exist in the same animal at the same time in a functional condition; the transplanted testicle in a female was not functional in the sense that it was producing spermatozoa, for there is no case on record known to the writer in which a transplanted testicle of a mammal produced spermatozoa. But in such a case the absence of spermatozoa is not the result of secretions from the ovary. In the opposite case, however, a male carrying two large ovarian grafts, that had been present for longer than eight months, was used for breeding purposes, and histological sections showed that the testis was actively producing spermatozoa. The two ovarian grafts in this male each contained many normal Graaffian follicles in all stages of development, from the primoidal follicle stage to the stage of maturation; there were many atretic follicles present but in one of the grafts more than seventy follicles possessing typical stratum granulosum and a well-rounded, normal-appearing ovocyte with typical dark-stained nucleus, proves that the ovary was functional. Certainly, in this case, the presence of the normal functional testicle did not prevent the growth of the ovarian graft, nor did it prevent the process of follicular development continuing, even though the graft had remained subject to any possible antagonistic influence for an extensive period of time relative to the sexual life of the animal. And it is just as true that the presence of the two functional ovarian grafts did not prevent the continuation of active spermatogenesis in the testicle of the male host, nor did it afford any evidence of suppression of the psychical nature of the male; the latter remained a typical male in all respects and was used for breeding purposes. The idea of sex-gland antagonism at least so far as the rat is concerned, is based upon insufficient and negative evidence, and in the light of positive evidence to the contrary must be considered untenable. The facts noted in the following pages, from observations made on guinea-pigs, shows also that the idea receives little support from a study of the effects of gonad transplantation in this animal.

<sup>3</sup> One gland was removed from the host to provide graft material for other operations, but that the function of the remaining gland was not impaired is proved by the fact that females bearing testicle grafts gave birth to young and that males bearing ovarian grafts were used for breeding purposes.

## MATERIAL AND TECHNIC

The technic employed in gonad transplantation has been adequately described in the preceding papers of this series. It is sufficient here to state that the normal sex glands were removed from the guinea-pigs (total castration) at an age of ten to twenty days after birth and that portions of the gonad from the opposite sex were grafted subcutaneously or intraperitoneally. In some animals two, three, or even four transplantations were made at intervals of five to ten days.

The fact that transplantation of gonads in guinea-pigs was less successful than in rats was mentioned in an earlier paper; in some of the guinea-pigs as many as four successive grafts have been made on the same animal without any of the grafts persisting. Over fifty guinea-pigs have been used in this series of experiments, but only a few examples will be given to illustrate the results.

## BEHAVIOR OF CASTRATED MALES AND SPAYED FEMALES

The most obvious result of castration of the male in the majority of animals is the total loss of all sex inclinations, if not at once, at least within a short period of time. However, exceptions to this general rule have been encountered many times in male guinea-pigs. Castration was performed by opening the peritoneal cavity, retracting the testicle into the abdominal cavity, tying off the spermatic cord with surgical silk thread to prevent bleeding from the internal spermatic artery, and cutting the cord considerably above the testicle, removing this organ entire. It is absolutely certain that in all cases the entire testicle was removed.

The castrated male animal, however, in many cases after a period of four to six months continues to exhibit the psychological characteristics of the normal male in that it utters the male sex call when a strange animal is placed in its cage, and many times will follow the female and even attempt to fulfill the normal functions of the male. This has been observed in many cases, but with these different individuals, as indeed with normal males, some are more vigorous in their reactions than are others; at times the reactions are so vigorous that one not acquainted with the condition of the animal would unhesitatingly take it for a normal male animal.

In the case of the female there are no reactions that can be considered male or female after the ovaries have been removed. In the normal female there are few elements in its behavior that are characteristically female with the exception of the reactions during the period of heat, or the reactions to its own young.

#### OBSERVATIONS OF EXPERIMENTS

##### *A. Males with ovary grafts*

Male 39 A, born April 24, 1918. May 11th testicles removed, two pieces of ovary transplanted subcutaneously on abdominal wall. February 15, 1919, killed. No testis tissue present. Sperm sacs long, but slender, not distended.

About one month after the transplantation of the pieces of the ovary, the teats of the mammary glands began to be noticeably increased in size and continued to increase until they reached the size of a pregnant female near term. The animal was confined alone in its cage until there was no doubt that the ovarian grafts had persisted and that the mammary glands resembled distinctly those of a normal female. Observations on the behavior of the animal were begun and continued over a period of some months, being conducted daily at some periods and intermittently at other times when the animal had remained alone for a period of about a week. The following observations will serve to represent the behavior of the animal for a period of three months:

November 2nd (five months after operation). At this time the teats of the mammary glands are at the height of their development, being as large as those of a pregnant female. Ovarian grafts on abdominal wall easily palpated. Young guinea-pigs placed in the cage, but upon attempting to suckle, the male fights them away and exhibits no sign of female psychical tendencies. When mother of young placed in cage, male follows her continuously, uttering male sex call and reacts so typically male-like that even copulation is attempted. When normal male is placed in cage with castrated male bearing ovaries a fight follows almost immediately.

November 7th. Female in heat placed in cage; the animal reacts as does a normal male though somewhat less vigorously.

November 11th. Another female in heat introduced into cage; same results as above; the animal utters the characteristic male call and attempts to imitate the functional male.



November 12th. Mother with two young placed in cage; mother removed periodically: young attempt to crawl under abdomen of male in search of teats, but the latter avoids them, or encircles them uttering male sex call; no sign of female tendencies. When mother replaced in cage, male follows her as would a normal male. The only indication of femaleness is the large, well-developed mammary glands (teats).

November 16th. Young remain in cage five hours; watched frequently during the time: no inclination toward young. Young attempt to crawl underneath abdomen, but male avoids them; no maternal inclinations. Mother upon being replaced in cage is followed almost as consistently as would have occurred in presence of a normal male.

November 23rd. After young had remained for some hours in cage with male, mother is introduced and young suckle her immediately; after few moments mother quickly removed and young, very excited, rush to male attempting to suckle; vigorous attempts, but male fights them away; no sign of female tendencies toward young. Mother reintroduced, male follows her immediately; typical male reactions.

February 12, 1919. Male still reacts characteristically as a male, following females as would a normal male. Teats are not so prominent at this time, but have become greatly reduced from the condition of November and December. At this time the ovarian grafts cannot be palpated and the grafts have evidently been resorbed.

February 15th. Animal killed. No grafts could be found and only small amount of scar-like tissue marks site of graft. Tissue removed from the site and sectioned.

Sections of the tissue removed showed that all the original ovarian tissue had undergone degeneration and resorption. The mass contained a great amount of fibrous connective tissue, but all of the characteristic ovarian stroma had been replaced.

In this animal the ovarian grafts became vascularized, had grown, and were noticeably present for a period of five or six months, and as long as the grafts could be palpated the teats of the animal were very large. In this respect the animal had developed perfectly distinct female somatic characteristics, and it is an interesting fact that after the grafts had undergone regression the teats of the animal also reverted to the type of the undeveloped structure characteristic of the normal male or castrated female.

The psychological characteristics of the animal, however, were not modified by the presence of the ovarian grafts; the animal retained the psychological disposition of a male throughout the entire period that it was under observation, though its reactions were



less vigorous than those of the normal male. There was never any doubt that the behavior of the animal was typically masculine and no single element of its behavior ever suggested feminine instincts. The animal exhibited no interest in young guinea-pigs, even at the time the mammary glands were at the height of their development, and would, indeed, fight them away when they attempted to suckle.

Male 40 A I., born April 29 1918. May 18th, testes removed, two pieces of ovary ingrafted subcutaneously. March 3, 1919, animal killed.

The ovarian transplantation was successful in this case; the glands were prominent and easily palpated in their position on the ventral abdominal wall. The teats began to be noticeably increased in size six weeks or two months following the operation, and by November had reached a size as large as those of a pregnant female near term.

November 2nd. Teats especially large, size of a suckling female. Young introduced into cage evokes no feminine reactions; attempting to suckle they are avoided or actually repelled by the animal. The male, though castrated and bearing two large ovarian grafts, reacts as a normal male; it follows the mother of the young continuously when she is admitted to the cage.

November 7th. Female in heat placed in cage evokes typical male reactions from the castrated male; male-sex call and behavior of the animal would lead one to assume at once that the animal was a normal male.

November 11th. Has remained in a cage with a spayed female for three days, yet upon introduction of a strange female the male reactions are noticeable at once; follows female uttering male sex call.

November 15th. Mother and young placed in cage; male reactions. Mother removed for three hours, but during her absence there were no indications whatever of a female psychical disposition: no maternal inclinations toward young; avoids young as they attempt to crawl underneath abdomen in search of teats. When mother is replaced the animal follows her immediately, uttering male call.

November 16th. Young placed in cage five hours, no feminine reactions. Mother placed with young long enough to begin suckling and is quickly removed; the excited young ones rush to male, attempting to suckle, but are fought away by the male (the mammary glands are at the height of their development). Mother replaced, animal immediately reacts as a typical male.

This same behavior continued throughout the entire period of nine months, and though observed many times the reactions were in no respects different from the above. At some periods the animal was tested daily or many times a day, and at other times at week intervals, the animal remaining alone in the meantime. In the case of this animal, as with several others, newly born young and their mother were allowed to remain in the cage with the observed animal for a week or longer so that the animal would become accustomed to the young, yet throughout the entire period there was never an indication of a maternal reaction on the part of the male. On the contrary, the animal retained its male psychical characteristics and reacted as a typical male. Before the animal was killed the mammary glands were noticeably reduced in size from their former swollen condition. The grafts were also somewhat less easily palpated than during December, and it was evident that regression was taking place. The animal was killed in March, 1919, at which time the grafts were almost indistinguishable; two small pieces of tissue, however, were cut from the site of transplantation and preserved in Bouin's fluid.

One of these grafts had been entirely absorbed, for there was no recognizable ovarian tissue in the scar-like mass cut from the site of implantation; muscle tissue and a great amount of fibrous connective tissue composed the entire mass.

The second graft, much the larger of the two, consisted of about six hundred sections of tissue, but the tissue was in a very degenerate condition. Cortex and medulla could not be easily distinguished. There were five or six recognizable Graafian follicles, but all of these were in a very degenerate condition the follicular cavities containing cell debris and the stratum granulosum undergoing dissolution. Throughout the entire mass, fibrous connective tissue was present in abundance, and it would be difficult to recognize the majority of sections as having been an ovary.

Male 68 A I. Born June 20, 1918. July 8th, testes removed, ovaries transplanted. February 15, 1919, animal killed.

Transplantation of ovaries into this male animal was so successful that the ovaries grew, could be easily palpated, and their position noted by a slight elevation of the skin over the site of the growing graft. Associated with the presence of the grafts, as in the preceding cases, teats had developed into large, well-rounded structures as large as those of pregnant females. The animal was tested periodically as in the cases already described, and the results were the same as those with the other ovarian grafted males. The animal, though possessing well-developed teats and the two large grafts, retained its male psychological characteristics throughout the entire period of the observations. It would follow the females, uttering the male call and otherwise behaved as a typical male. And, as in the former cases, the animal exhibited no maternal or feminine instincts throughout its entire life; it would fight away young ones that attempt to suckle it.

At the time of killing, the teats of the mammary glands were very prominent; the grafts were recovered from their original site of implantation and preserved in Bouin's fluid.

Both of the ovarian grafts had persisted for the seven months after transplantation, but histological sections show that the smaller of the grafts consists largely of fimbria of the oviduct. There was but little ovarian stroma or included cells (considered as interstitial cells) in the graft and no normal Graafian follicles were observed.

The larger graft consisted of characteristic ovarian tissue as well as a small part of the oviduct and fimbria. The peritoneal capsule that normally surrounds the ovary had been removed during the process of transplantation, and in so far as the epithelial covering of the ovary was not protected from the tissues of the host, the tissue of the ovary grades almost imperceptibly into the surrounding connective tissue and muscle.

There were more than 500 sections of the graft that contained typical ovarian tissue (cut  $10\mu$  thickness). Within this tissue are many Graafian follicles, some undergoing atresia and others that are normal. The normal follicles are young ones (primordial follicles) or older ones that show a typical follicular cavity, and all other elements of the normal follicle in the usual relation.



In this case the ovarian grafts after seven months are composed of characteristic ovarian tissue. The somatic modification brought about during its growth in the male was distinct, inasmuch as the teats of the animal, which ordinarily remain rudimentary in the male, had increased in size and were apparently as fully developed as those of a pregnant female. The psychical modifications, however, were entirely negative; the animal showed no feminine inclinations throughout the entire period of its life, and though possessing large teats it would repel any attempts of young animals to suckle. Here, then, the gonad is associated with the development of one of the 'homologous' characters but there is no evidence that 'heterologous' characters were inhibited.

Male, 72 A. March 27, 1919 (approximately thirty days old), testes removed. Two ( $\frac{1}{2}$ ) ovaries grafted intraperitoneally, two ( $\frac{1}{2}$ ) ovaries subcutaneously, ovaries from female of same age. April 19th, second operation repeated as above, ovaries from nineteen day female.

In the course of six weeks or two months after the ovarian transplantation the teats of the mammary glands of the male increased remarkably in size, and by the end of July compared favorably in size with those of a pregnant female near term.

Observations on the behavior of the animal were made during a period of three months, and its reactions were found to differ in no essential manner from the reactions of the animals described above. The psychical characteristics were typically those of the male: interest in strange animals placed in the cage, the sex call of the male, and attempts at copulation were the reactions observed and interpreted as male-like. The reactions, however, were somewhat less vigorous than the usual normal male reactions. Tested with young guinea-pigs, the male showed no reactions that could be used as an indication of femaleness: not only did the animal fail to respond to the young with a maternal attitude, but it would fight away the young ones when they attempt to suckle, and this at the time the mammary glands were at the height of their growth.

The animal was killed November 15, 1919, but at this time the mammary glands had undergone some regression. Figure 1 A is a photograph of the animal taken two days before killing, to



show the condition of the mammary glands eight months after the original ovarian transplantation. Figure 1 B shows for comparison the same structures of a normal male animal; in the latter the teats, one on each side, are scarcely visible, while on the operated male they are very prominent though reduced somewhat in size from their condition of two months earlier.

As indicated above, eight pieces of ovary were grafted into the animal in two separate operations.<sup>4</sup> Before death two subcutaneous grafts could be palpated and after death these two subcutaneous grafts as well as two intraperitoneal grafts were recovered from the animal; vascularization was well established in all.

### *B. Females with testis grafts*

In transplantation of pieces of guinea-pig testis a smaller number of grafts grew or persisted for an appreciable length of time, but the effects of the grafts were somewhat more apparent than in ovarian transplantation, especially when considering the accompanying psychical effect. Following simple ovariectomy, the female guinea-pig is, without exception in my experience, sexually indifferent; such an animal's reactions give no indication of maleness or femaleness. Accompanying growth of testicular grafts in a completely spayed female, however, the animals, as they become adults, react typically as males both toward each other and toward other animals.

Though testicular transplantation was made in fifteen or eighteen female guinea-pigs following complete ovariectomy, in some cases of which the animal received three or four successive grafts at intervals of from five days to a week, there were but two animals in which the grafts persisted long enough to study their effect. In each of these animals (67 B1 and 68 B1) a single operation was made fifteen and eighteen days after birth, respectively, when the ovaries were removed and two pieces of testis grafted subcutaneously. Animals 67 B1 was killed March 3, 1919, nine months after the transplantation was made, and both grafts,

<sup>4</sup> The young ovary, used as material for the grafts, was cut into two pieces with scissors, so that in all four ovaries were implanted in this animal.

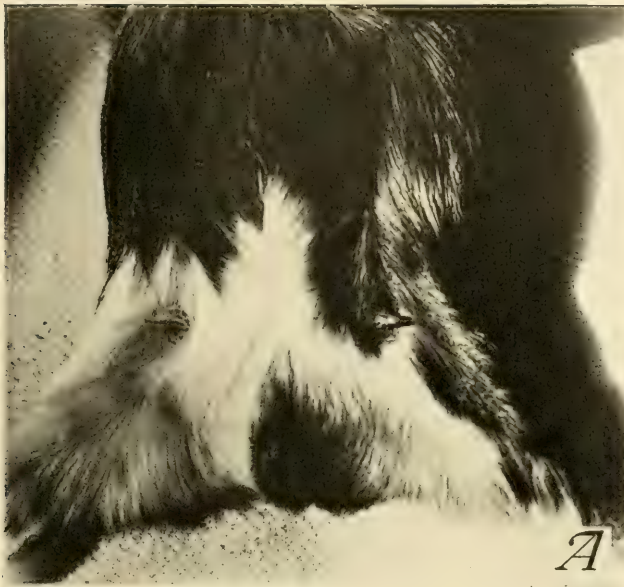


Fig. 1 A<sup>5</sup> Photograph of ovarian grafted male guinea-pig (72 A), eight months after implantation of ovary, showing hypertrophied condition of teats.

Fig. 1 B Photograph of normal male guinea-pig showing normal condition of teats (compare with fig. 1 A).

<sup>5</sup> Drawings and photographs by Kenji Toda.

now of large size, were recovered, preserved, and sectioned for study. Animal 68 B1 was killed February 15, 1919, seven months after the original transplantation, and the two grafts likewise recovered, preserved, and sectioned.

The psychical reactions of each of these animals had been those of a typical male for some months before they were killed. Upon introduction of a strange animal into the cage of either of the two, the animal became very excited and began to emit the characteristic call of the male animal as it approached the strange one. If the latter animal was a female the psychically changed female attempted to imitate the copulatory reactions of the normal male animal, while if the animal introduced was a male a fight usually began. In the male-like reactions each of the two animals were decidedly vigorous, and one observing their behavior would conclude at once that the animal was a normal male.

The somatic characters that distinguish a male guinea-pig from a female are indeed few, but the configuration of the external genitals is one distinct feature that serves as a ready means of identification of the two sexes. And inasmuch as the development or differentiation of the external genitals are not entirely completed fifteen days after birth, they would be subject to influences of the implanted sex gland.

In each of the two individuals decided modification of the configuration of the external genitals occurred. The clitoris of the female underwent considerable hypertrophy, producing a prominence that externally resembled very decidedly the male penis. As a result of this the general configuration of the genital region resembled the male condition considerably more than the female.

Lipschütz, observing one of Steinach's transformed females, has written several papers describing the modification, and inasmuch as the transformation in the animals of my series differs in no material respects from that described by Lipschütz, further description of the conditions will be omitted.

Observations on the two female guinea-pigs of my series confirms the findings of Steinach that testis grafts will persist and grow in the female, and that associated with this growth the female may develop a typical male behavior toward both



females and males (psychical modification) and that the external genitals of the female may undergo such changes that they come to resemble the male condition more than the female (somatic modification).

In animal 67 B1, the testicle graft reached a large size, both grafts having persisted for slightly longer than nine months when the animal was killed. A rough measurement of the excised grafts, 10 x 7 x 5 mm., represents the entire mass of the tissue, but part of this tissue consists of encapsulating connective tissue and muscle. However, after fixation, paraffin embedding, and sectioning, each of the two grafts consisted of over 600 sections of testicular tissue (cut 10  $\mu$  thickness).

The tissue is remarkably well preserved for a testicular transplant, as the graft consists of compact testicle tissue within which there is no evidence of necrosis. The principal part of the tissue is the region of seminiferous tubules, but in one graft there were approximately 200 sections containing an area of epididymis about one-sixth of an entire cross-section. The other graft consisted of a smaller amount of epididymis. This part of the tissue is characteristic of the testicle and need not concern us further.

The seminiferous tubules were compactly arranged and the intertubular spaces were no larger than under normal conditions (fig. 2). Associated with this the interstitial cells are not more abundant than is ordinarily found in the testicle. In other words, there is no indication of an hypertrophy of the interstitial gland in either of these two grafts. The germinal epithelium has suffered some degeneration, as there is an entire absence of spermatozoa, spermatids, and in many places of spermatocytes. However, there has not been so great a degeneration of the germinal epithelium as is usually the case, for the tubules contain in many places an epithelium three or more cells in thickness. Many karyokinetic figures occur in the epithelium lining the tubules, and there are also many cells lying free in the cavity of the tubules.

The grafts from animal 68 B1 differ somewhat from those of animal 67 B1. In animal 68 B1 the grafts persisted longer than seven months, and are somewhat different in size. The larger



graft consists of about 570 sections ( $10\mu$  thick), the smaller one of over 450 sections. The larger graft is composed of about equal amounts of seminiferous tubules and epididymis tissue, while the smaller is made up almost entirely of seminiferous tubules.

Figure 3 is a cross-section of the larger subcutaneous graft and shows both epididymis and seminiferous tubules as well as the

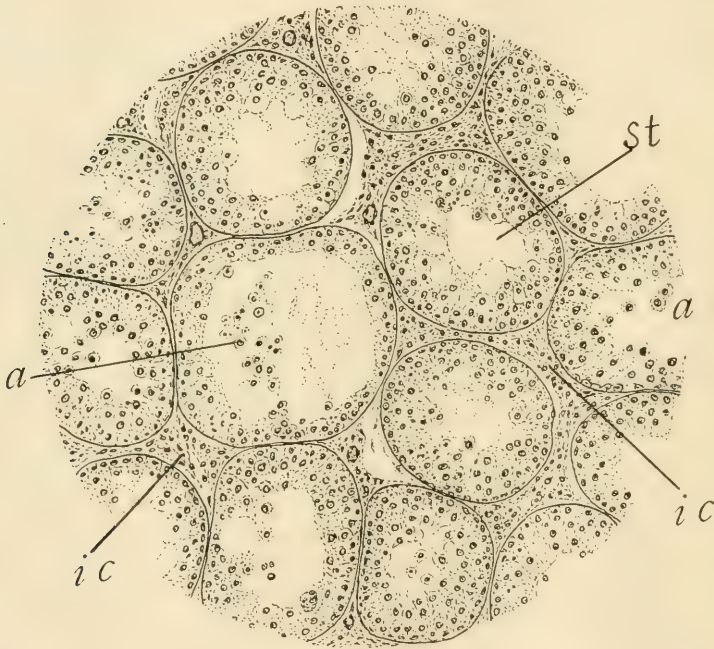


Fig. 2 Part of section of testis graft from female guinea-pig (67 B 1) nine months after transplantation. *a*, cells free in seminiferous tubule; *ic*, interstitial cells; *st*, seminiferous tubule.

character of the tissue surrounding the graft. The epididymis tissue is characteristic of the testicle under normal conditions, except that it does not contain spermatozoa. The seminiferous tubules, however, have been modified; the entire mass of germinal epithelium has disappeared, with the exception of a single layer of cells next the basement membrane that are considered as Sertoli cells. The tubules, though devoid of all cellular material

other than that just mentioned, remain well distended, with no tendency to collapse (fig. 4). Within the tubule is a reticular-like mass of material that possibly represents the old stroma of the germinal epithelium, but no nuclei can be seen.

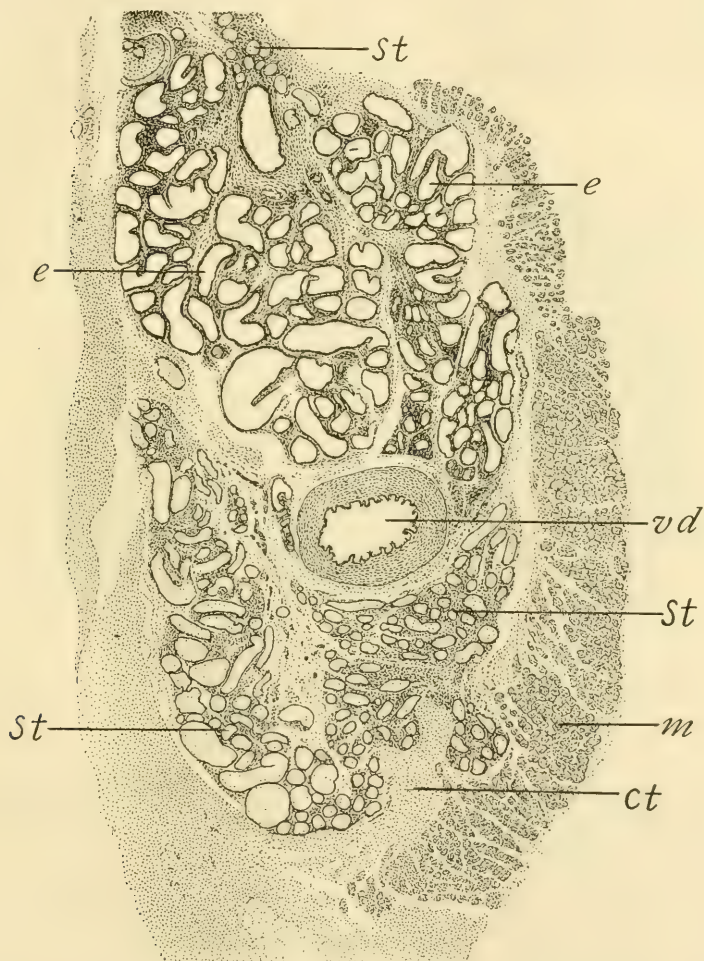


Fig. 3 Entire cross-section of testis graft from female guinea-pig (68 B 1), seven months after transplantation. *ct*, connective tissue; *e*, epididymis; *m*, muscle tissue surrounding the graft; *st*, seminiferous tubules; *vd*, vas deferens.

The tubules are not so compactly arranged as in the normal condition, but are widely separated and the intertubular spaces are filled with interstitial cells. These cells are distinct, well stained, and large, and apparently there has been an hypertrophy of the interstitial tissue. The cells are present in an abundance, filling completely the large spaces between adjacent tubules. The conditions are relatively the same for the two grafts.

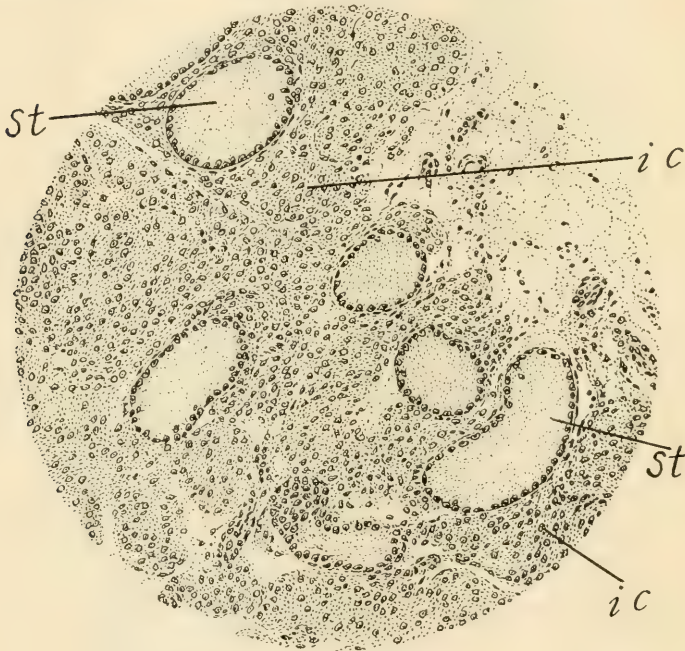


Fig. 4 Part of cross-section of testis graft from female guinea-pig (68 B 1), seven months after transplantation (same animal, but different graft from fig. 3). *ic*, interstitial cells; *st*, seminiferous tubule.

The reason for the difference between the grafts from the two animals is not clear. That of 67 B1 has persisted for nine months and was slightly larger than in 68 B1: the germinal epithelium of the former was not nearly so much affected as in the latter and the tubules were more compactly arranged: there was little or no increase in the interstitial cells of the former, while in 68 B1 the tubules are widely separated by a very prominent mass of inter-



stitial cells. The grafts of 68 B1 had persisted for seven months, and, as indicated, consist principally of interstitial cells; the germinal epithelium has entirely disappeared, leaving only the Sertoli cells. The condition of this graft evidently represents the conditions so often described as an hypertrophy of the interstitial gland.

The relative effects of the grafts upon the somatic structures are essentially the same in the two animals; if any different, the the external genital condition was more modified in 67 B1. In the psychical reactions, also, the latter was a more vigorous male-like animal than was 68 B1. And since a slightly more pronounced modification was associated with the graft in which the interstitial cells were present in lesser abundance it does not lend support to the idea that the interstitial cells are the seat of the elaboration of an internal secretion upon which the modifications depend.

#### DISCUSSION

In former papers the writer has taken a view-point that is decidedly at variance with the idea of an antagonism existing between the two opposite sex glands, the chief supporter of which is Steinach.

In view of the great number of researches upon the modification of the secondary sex characters of vertebrates by castration experiments and sex-gland transplantation, there is no doubt that many of the characteristics of the different sexes are dependent upon the integrity of the sex gland for their existence. Thus from ancient times the effects of the removal of the testicle from a young human individual has been exemplified by the eunuch. And to mention but two or three more recent researches, Stotsenburg has shown that the presence of the ovary in female rats is responsible for the relatively lighter weight of the animal. In the rat, at least, the testicle has no influence upon the growth curve. The weight character is then influenced by one sex gland alone. And in an entirely different class of vertebrates Pezard ('18) has given an excellent analysis of the influence of the sex glands in modifying the somatic and psychical character of the ani-



mal. In the common fowl one ordinarily considers the spurs and typical feathering of the cock as secondary sexual characters due to some influence of the testicle. Pezard has shown, however, that these are secondary sex characters of the cock only because the development of these structures has taken place in the absence of an ovary. A castrated cock or an early spayed pullet develop the typical cock feathering and spurs as do normal males, hence their presence does not depend upon a secretion of the testicle. They are potential characters of both sexes and their absence, normally, in the female is due to some effect of the ovary.

Steinach has neglected fundamental conditions of this character in arriving at his general conclusions. Thus when a testicle was grafted into a spayed female rat (or guinea-pig) and the animal became relatively heavier than other females, Steinach claims the results point to an effect of the testicle secretion and uses it as partial proof of the masculinization of the female animal. However, the relative increase in weight is due only to the removal of the influences of the ovary and not to any influence of the testicle, for the testicle has no influence upon the growth curve. This character is comparable to spurs and cock feathering in the fowl which are also not affected by the presence or absence of the testicle, and I suspect that the same is true in regard to weight in the guinea-pig.<sup>6</sup>

There appear to be not more than two distinctive somatic characters of a male or female guinea-pig that are capable of modification by sex-gland transplantation. These are, (a) the teats of the mammary glands and, (b) the configuration of the external genitalia. Rudimentary teats are present in both animals and remain in an undeveloped condition in both the normal male and the spayed female. In the normal female the teats grow as sexual maturity is reached and undergo considerable hypertrophy during pregnancy, and as Steinach's and my own experiments show, they will increase in size in a castrated male animal in which ovarian grafts are growing, until they reach the size of those of a pregnant female. This somatic modifying power of the ovary is

<sup>6</sup> Experiments are now under way to determine the effect of castration and spaying in the guinea-pig, in reference to its growth curve.

unquestionable. And also the external genitals of the female are capable of modification as the result of the growth of a testicle graft in a spayed female. Steinach was first to point out this modification, and Lipschütz had described the modification wrought in the case of one of Steinach's animals. The clitoris becomes hypertrophied until the configuration resembles a penis-like structure, and the appearance of the genitals as a whole resemble the condition of the male animal more than that of the female. Aside from these two modifications, there are no features of a somatic nature that the writer can use as sex differentials. A priori, if the weight of a normal female is less than that of the normal male, and spaying results in a relative increase in weight of the female (as is the case in rats), one would naturally assume that a growing ovary in the male would relatively reduce its weight. It may be possible that such is the case, but for this character to be of any use as a sex differential would necessitate a great number of cases in which a good ovarian graft was present.

The weight of different individuals of the same age is such a variable quantity that random comparisons are of no value. If the weight conditions in guinea-pigs are comparable to those of rats, we know that the presence of a testicle or testicle graft would have no influence on the weight of the animal. Yet Steinach and Holzkecht ('17) publish a table of weights of three guinea-pigs (norm. female 845 grams, norm. male 1002 grams, masculinized female 1200 grams) which indicates that not only did the testicle graft cause a relative increase in weight of the female, but the female became more of a male than the normal male itself. Likewise, another table of weights (norm. male 980 grams, norm. female 808 grams, feminized male 516 grams) indicates that an ovarian graft changes a male into an animal more feminine than the normal female. The writer is inclined to consider such a condition as very confusing and to discredit the data as evidence of sex-gland activity.

Many difficulties are involved in an intelligent analysis of the psychical nature of animals and there is very great danger of the personal equation influencing an interpretation. On the male side the animal is always aggressive when strange animals are intro-

duced and there is an almost, if not an entirely specific sex call that a male utters when approaching the female; there is also the characteristic copulatory reaction which is specific for the male. On the female side there are no positive reactions other than the characteristic periodic heat reactions and the maternal behavior toward the young.

From this series of experiments the observations of the female animals bearing testicle grafts leads one to conclude that the psychological behavior of the female has been changed in the direction of that of the normal male; its reactions toward other animals is typical for the male. But in the case of the male there was never any indication that female psychological tendencies had been brought out. The writer does not wish to assert that it is impossible to attain such modifications, but in males in which the mammary glands had reached the height of development characteristic of a pregnant female no such reactions could ever be observed. All tests of the ovarian grafted male have been negative in regard to maternal instincts.

In the guinea-pig there are few structures that are capable of throwing any light upon the question of sex-gland antagonism, except the possibilities of growth of both gonads in the same individual. Sand ('19) has shown that such a condition is possible; therefore, the indications are direct that no such antagonism exists. But as to an antagonism in reference to the psychological characteristics, though usually the animal loses all sex tendencies, yet in some cases even though the male animal possesses growing ovarian grafts that are effective in causing an hypertrophy of the mammary glands, the animal continued to react psychologically as a male animal. And if there is an inhibitive effect of an ovary on the male psychological nature, surely it would have been in evidence in this place.

In the final analysis the writer considers that there is an entire lack of evidence of an antagonistic influence of the sex glands. In the guinea-pig an ovarian graft does not inhibit the male psychological influence, and this is all that it has a chance to inhibit, excepting possibly that it may be the factor that causes the female animal to be relatively lighter in weight than a male. The evi-



dence for this, however, cannot be supplied by a random comparison of one animal with another. There is no indication that the mammary glands are inhibited by a testicle growth, for if the ovary be removed they remain in a rudimentary condition as in the normal male or spayed female. Also in the rat, there is not only an entire lack of evidence of an antagonism, but there is positive evidence to the contrary. Ovaries and testes grow in the same animal in a functional condition: the testicle does not inhibit the growth of an ovarian graft, neither does an ovary prevent the growth of a testicular graft. The ovarian graft does not inhibit the growth of the sperm sacs, the penis, the functional condition of the testicle, or the psychical nature of the animal.

The positive modifying effects of the gonads in cases of transplantation to an animal of the opposite sex are: 1) in the rat there is some evidence that the psychical nature of the animal undergoes some change toward the sex represented by the gonads, though this is difficult of intelligent interpretation; the presence of the ovary, shown in cases of spaying females, causes a slight relative reduction in weight, and, 2) in the guinea-pig an ovary grafted in the male can cause a hypertrophy of the mammary gland, and possibly influence the psychical nature of the animal, though my own experiments give no indication of this: a testicle graft in a female can cause the development of the male psychical tendencies, and also may influence the growth of the clitoris until the external genitals appear more like the male condition than the female. In reference to these positive characteristics the writer is in agreement with Steinach, but upon the basis of observations on more than a hundred operated animals the writer can only conclude that there is not only lack of evidence of an antagonism existing between secretions of the sex glands, but there is considerable evidence to the contrary; and not only evidence, but positive proof, inasmuch as the two opposite sex glands have remained functional in the same animal for a period of from seven to nine months.



## SUMMARY AND CONCLUSIONS

1. Pieces of ovaries implanted in a young castrated male guinea-pig will grow and retain the characteristic ovarian tissue for a period of several months.

2. The presence of ovarian grafts in the male leads to an hypertrophy of the teats of the mammary glands to such an extent that they resemble the conditions of a pregnant female (somatic modification). The psychical characteristics of the male, however, were unmodified; such animals gave no indication of acquired feminine instincts in reference to young animals: they not only avoid the attempts of young to suckle, but fight them away.

3. Testicular transplantation into young spayed females, though successful in a less number of cases than ovarian grafting, is possible. The testicle tissue remained in a typical condition for nine months after transplantation, except that spermatozoa are absent from the tubules.

4. The effect of the testicle tissue in both somatic and psychical modification was pronounced. The clitoris of the female underwent hypertrophy resulting in a condition resembling the male more than the female (somatic modification). Such animals behaved as typical males toward each other and toward females (psychical modification).

5. The writer considers that there is an entire lack of evidence of an active antagonistic effect between the secretions of the two opposite sex glands.

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Resumen por el autor, George Howard Parker,  
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El poder adhesivo de las ventosas de *Octopus bimaculatus*  
Verrill.

Las ventosas extirpadas de los tentáculos del cefalópodo *Octopus bimaculatus* pueden ejercer una presión que varía de 45 a 70 por ciento de atmósfera, cuando se las estimula eléctricamente.

Translation by José F. Nonidez  
Cornell Medical College, New York

## THE POWER OF ADHESION IN THE SUCKERS OF *OCTOPUS BIMACULATUS VERRILL*<sup>1</sup>

G. H. PARKER

ONE FIGURE

During the summer months *Octopus bimaculatus* can be found abundantly in the rocky tidal pools in the neighborhood of the Scripps Institution for Biological Research at La Jolla, California. When this animal is picked up it commonly attaches itself to the hand and fingers of the collector by its suckers, thus producing a strange and almost uncanny sensation. Not only will the whole animal suck to the hand, but an excised arm will exhibit coördinated movements and vigorous suction, and even an isolated sucker, when stimulated electrically, will hold to the finger of the experimenter apparently with as much vigor as when it was a portion of the whole animal. These parts, therefore, exhibit a very unusual degree of autonomy and, since the isolated suckers are very conveniently handled, they afford excellent material on which to test the power of suction.

Freshly excised suckers were suspended by a strong thread to a hanging spring-balance. The sucking disc, which faced downward, was then applied to a piece of wet smooth wood and the sucker brought into action by stimulating its base with a faradic current. The electrodes by which the current was applied were manipulated by one hand of the experimenter while by the other hand the piece of wood, to which the sucker had become attached, was lowered till the suction was overcome and the wood and sucker parted. Meanwhile the observer watched the indicator on the spring-balance and noted the point indicated on the scale when the parting occurred. This point gave the breaking force involved and was read in grams. As a rule, four or five such read-

<sup>1</sup> Contributions from the Zoölogical Laboratory of the Museum of Comparative Zoölogy at Harvard College. No. 331.



ings could be obtained from each sucker. As it was the object of this investigation to ascertain the maximum capacity of each sucker, the highest breaking force among the four or five observed was taken rather than an average.

The faces of the suckers in *Octopus* are very regularly circular and after each test the diameter of the given sucker was measured in millimeters. From these diameters the areas of suction of the several suckers were calculated in square millimeters. The theoretical maximum suction for each such area was then worked out on the assumption that an atmosphere is equal to 1.033 kilo-

TABLE 1

*Diameters of suction areas (millimeters), calculated areas of suction (square millimeters), breaking forces (grams), theoretical maximum suction (grams), and percentages of efficiency for eight suckers from the arms of Octopus bimaculatus*

	NUMBER OF SUCKER							
	1	2	3	4	5	6	7	8
Observed diameters of suction areas in mm.....	2.3	2.8	3.0	3.9	4.5	5.2	5.5	6.0
Calculated areas of suction in sq. mm.....	4.15	6.16	7.07	11.95	15.90	21.24	23.76	28.27
Observed breaking forces in grams.....	29.2	42.5	51.0	62.4	79.4	99.2	122.1	147.4
Theoretical maximum suction in grams.....	42.9	63.6	73.0	123.4	164.2	219.4	245.4	292.0
Efficiency in percentages..	68	67	70	51	48	45	50	50

grams per square centimeter. These derived results together with the original observations are brought together in table 1.

In table 1 the suckers have been arranged in the order of size from smallest to largest. The smallest one shows the smallest breaking force, 29.2 grams, and the largest one the largest, 147.4 grams, the others forming a series between these two extremes. This series, however, does not conform very closely to the series of theoretical maximum suction calculated for the series of suckers on the basis of atmospheric pressure. The relation of these two series is more easily understood from the plottings in figure 1 than from the numbers in table 1. The

theoretical maximum suction calculated for the eight suckers is shown by the dots in curve B. The observed breaking forces for these eight suckers are designated by the crosses that afford the basis for curve A. At all points curve A lies below curve B and this relation is what should be expected, for it is quite impossible that a sucker acting purely as such should at sea level exert more than one atmosphere of pressure.

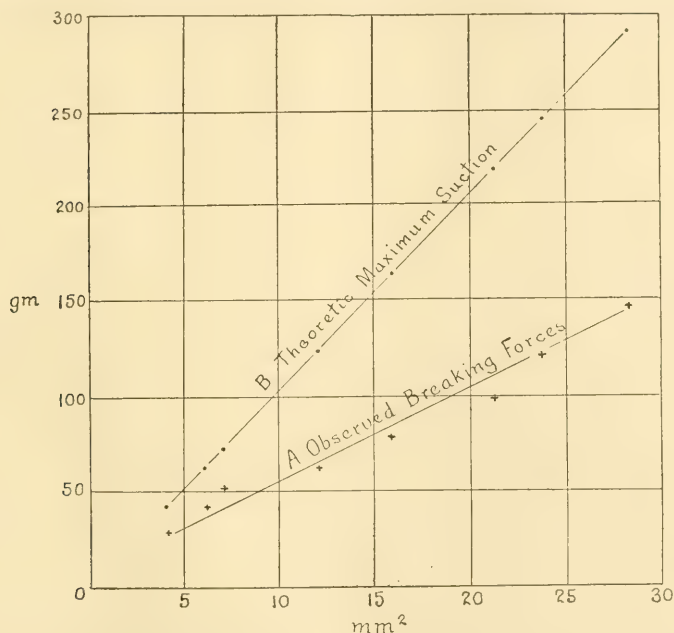


Fig. 1 Plottings of the observed breaking forces (A) and of the theoretical maximum suction (B) of suckers of different sizes (table 1); the ordinates represent grams, the abscissae square millimeters.

If in any sucker the breaking force were equal to the theoretical maximum, that sucker could be said to have an efficiency of 100 per cent. Such, however, is never the case, for, as is shown at the bottom of table 1, the efficiency of these suckers is never higher than 70 per cent of the maximum and may fall as low as 45 per cent, with an average on the eight readings of 56 per cent. This efficiency appears to be rather higher for the

smaller suckers than for the larger ones. But even in the smaller suckers it is well under the theoretical maximum and falls far short of the efficiency of the sucking organs of such animals as the sea-anemone *Cribrina* (Parker, '17), which can exert a pressure of about one atmosphere.

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Resumen por el autor, Chikanosuke Ogawa,  
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Experimentos sobre la regeneración del cristalino de *Diemyctylus*.

1. El autor describe con detalle la regeneración del cristalino en *Diemyctylus*.

2. Algunos de los experimentos se han llevado a cabo en la porción superior del iris. La porción inferior es incapaz de producir un cristalino, aun cuando se interrumpa la regeneración en la porción superior. Haciendo una incisión en forma de colgajo en la porción superior del iris, la regeneración que sigue tiene lugar de diferentes modos. Un trozo de iris transplantado en la retina puede provocar la regeneración del lente mejor que en la cámara del ojo.

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# EXPERIMENTS ON THE REGENERATION OF THE LENS IN DIEMYCTYLUS

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FOUR FIGURES

## INTRODUCTION

In embryonic development the lens is formed from the ectoderm covering the eye cup. If the larval or adult Urodele is experimentally deprived of its lens, regeneration takes place from the dorsal side of the iris, which has no relation with the normal embryonic development of the lens. This striking fact of heteromorphosis, which was first discovered by Colucci,<sup>1</sup> later led many investigators<sup>2</sup> to study this problem further from various points of view. Not only have descriptions of the regular regenerating process been given in detail, but also the factors that stimulate and determine the formation of the lens, the reason that the seat of regeneration is exclusively in the upper iris, and many other questions have been investigated.

In the present investigation the author has studied further the regular regeneration of the lens, and in relation to it has also made a number of experiments upon the dorsal part of the iris (upper iris).

## MATERIAL AND METHODS

Adult specimens of *Diemyctylus* were used exclusively as material for the present study.

The method of operation is as follows: The whole body of the animal, except the head, is wrapped in cloth. A piece of cloth is put into the mouth in order to keep the eye protruded.

<sup>1</sup> See Emery ('97).

<sup>2</sup> Wolff (larva and adult of *Triton taeniatus*), Fischel (35-mm. long larva of *Salamandra maculosa*), Müller (3- to 6-cm. long larva of *Triton*), Wachs (larva of *Salamandra perspicillata*, *Triton taeniatus*, *cristatus*, *axolotl*), etc.

Under the binocular, an incision is made into the nasal part of the cornea with a small iridectomy scapel and then the cut is extended horizontally. Flowing off of the aqueous humor can be prevented until the cut is finished by slight pressure of the back of the scapel against the wound; in this way prolapse of the iris is avoided. Compression on the eye causes the lens to slip out easily through the wound. The right eye alone was operated upon. After the operation, the animals were put directly into the water. The wound heals quite easily and the eye recovers its original form.

For convenience of description, I shall suppose the eye axis to be in sagittal direction.

#### RESULTS OF EXPERIMENTS

##### *Regular regeneration of the lens*

As already known, the first changes that take place after extirpation of the lens are depigmentation of the epithelial cells of the dorsal part of the iris and thickening of the upper iris edge. Concerning the point where depigmentation first begins, however, existing statements are not exactly in agreement. According to Wolff and Müller, "die innere Lamelle der Iris beginnt ihr Pigment zu verlieren," while Fischel says "die erste Veränderung besteht darin, dass die Zellen ihres hinteren epithelialen Blattes pigmentärmer werden und die Depigmentation greift auch über den Pupillarrand hinaus eine Strecke weit auf das vordere Blatt über." Wachs mentions simply that "die Umschlagstelle des äusseren in das innere Blatt entpigmentiert sich;" my observation agrees with this latter statement. The first change, pointed out by Wolff and Müller, corresponds to a slightly later stage.

The first depigmentation occurs usually not in the whole upper iris edge, but just in its middle part, only now and then being displaced laterally or medially. Simultaneously with depigmentation, or rather prior to it, the iris edge undergoes thickening and presents a club shape if seen in sagittal section. The thickening is caused by the splitting open of the two epithelial layers of this part and also by the increase of their height. Mitosis cannot be seen in this stage. Several round cells, richly provided

with pigment granules, appear in the space produced by the splitting of the two layers. These round cells were considered by Wolff and Müller as leucocytes. Concerning the separation of the two epithelial layers of the iris, Wolff is inclined to assume that it is caused by pressure of the leucocytes which enter between them. On the contrary, Fischel states as follows: "Mit einer einfach mechanischen Erklärung kann man hier nicht auskommen . . . . Die Zahl der Leukozyten ist eine zu variable und ihr Durchtritt durch die Iris erfolgt in zu unregelmässiger Weise und nicht an allen Stellen, um eine so gleichmässige, allseitige Abhebung bewirken zu können." It is not justified, however, to count the presence of the leucocytes as a cause of separation, because even in cases where no leucocyte is visible, separation of the two layers can be seen.

Thickening as well as depigmentation increases and extends from the middle part of the edge in various directions. The iris edge prevents a more distinct swelling. Especially cells of the inner layer increase in their height. Although the cells are arranged usually in a single layer in the beginning of regeneration, they may consist in this stage already of two layers. As a result of depigmentation, nuclei are revealed gradually in epithelial cells of both layers. While Wolff, Müller, and Fischel point out depigmentation in the inner layer, it takes place at first also in the outer layer in slight degree. The split between the two layers becomes more marked and extensive, and round pigment cells are to be seen in the cavity. As Fischel mentioned, depigmentation and thickening take place also in the lower iris edge in this stage and extend gradually toward other regions.

Still later, epithelial cells in the iris edge lose their pigment more and more, finally becoming entirely pigment free. The same process extends in the inner layer dorsally from the edge. Depigmentation in the outer layer, however, reaches only such degree that the nuclei become visible in the cell bodies. Sometimes the whole inner layer from the iris edge to the pars ciliaris may lose its pigment, but in other cases, on the contrary, total depigmentation is restricted to the iris edge and does not extend further, the rest of the iris containing more or less pigment all the time.



Between the pigment-free cells of the inner layer, occasionally a few round cells can be seen, which are larger than the iris epithelial cells and richly laden with pigment granules. It is difficult to decide whether they are leucocytes or modified epithelial cells, though the former assumption seems more likely. The upper iris, thus depigmented, elongates at the same time downward.

The upper iris edge becomes more enlarged by the increase of cell height as well as by the widening of the split, and thus forms a round vesicle. Mitosis is often seen here. The part which connects the enlarged iris edge with the original iris—the stalk—becomes gradually thinner and the split between the two layers here diminishes, so that the cavity of the iris edge does not communicate with the original split of the iris. Then the cells in the posterior pole of the vesicle grow higher and at the same time thinner in a radial direction. The adjoining cells also become successively high. In short, the cells in the posterior wall take up a concentric arrangement and protrude into the cavity of the vesicle. These are lens fibers. As the development proceeds, the cavity is narrowed by these lens fibers and finally disappears. Here a solid lens is formed, which is still connected by the stalk with the original iris. The cells constituting the stalk are flat or cuboidal and often small.

When the lens grows and attains a pretty advanced stage, pigmentation comes back gradually to the iris epithelium and the thickening also decreases. In this way the normal state is restored. Sometimes the pigmentation process in the iris epithelium is retarded, though the thickness returns to the norm or vice versa. The lens separates from the iris and remains in the pupillar region. The lens may either simply separate from it or, prior to separation, be connected with it by fibrous ligament. No consideration is given at present to the question as to how this ligament was formed. On the other hand, the lens may or may not become connected with the lower iris until it separates from the upper iris. This connection occurs directly or by fibrous ligament. The epithelium of the lens is sometimes pulled out where the lens is in connection with the iris, namely, about in the

equatorial zone. Owing to this projection, a cavity remains between the lens epithelium and its substance, though later both projection and cavity disappear. This connecting zone is variable in its position, deviating anteriorly or posteriorly in different grades from the equator; the position of the lens accordingly is also variable. According to Fischel, "die Art und Weise der Lösung dieser Verbindung lässt es zweifellos erscheinen, dass sie lediglich durch das Gewicht der wachsenden Linse erfolgt; hat dieses eine bestimmte Grösse erreicht, dann wird die Verbindung gelöst." But I think it is most unlikely that separation takes place by the growing weight of the lens, because the lens, after separation from the iris, still remains in contact with the iris in the pupillar region.

The lens capsule is formed at first on the anterior surface of the lens while the lens is in connection with the iris.

In normal embryonic development, pigment cells are found sometimes in the cavity of the lens vesicle; the same phenomenon is seen occasionally also in the regenerating lens. Besides the change in the iris epithelium, it is observed now and then that the gold pigment cells in the iris stroma extend to the surface of the lens.

#### *Time of regeneration*

Concerning the time required for the regeneration of the lens, Fischel ('00) states the following: "Ebensowenig glaube ich thermische Einflüsse eine Wirkung zusprechen zu können. Denn die Regeneration erfolgt im Sommer nicht rasher als im Winter, trotzdem der Temperaturunterschied des Wassers, in dem die Tiere gehalten werden, gewiss kein unbeträchtlicher ist." My results show, on the contrary, that the time required for regeneration is different according to the season and individual variety. For instance, thickening in the upper iris edge appears in five days after operation in summer, while it begins in nine days after operation in late autumn. Again, for the first appearance of the lens vesicle, it takes two weeks in summer and more than three weeks in autumn. Since the operated animals were all killed

before complete development of the lens, the exact time necessary for the whole process cannot be given here, In general, after the development of the lens attains a certain stage, further change is slow.

In order to confirm the possibility of regeneration in winter, ten animals were operated upon the middle of December, and the eyes were examined after forty days, but no indication of regeneration, such as depigmentation and thickening of the iris epithelium, could not be found at all. In this case the temperature of the aquarium, where the animals were kept, ranged from 7° to 4°C. Difference in duration of regeneration according to the season and absolute lack of regeneration in winter seem most likely to depend upon temperature, though food might play some part.

Individual difference in time of regeneration is fairly marked, even if the animals are kept under the same conditions; twenty specimens were studied three weeks after operation, and the results showed that the regenerated lenses are variable in size, the largest being several times larger than the smallest.

#### EXPERIMENTS ON THE IRIS

##### *Lack of regeneration from the lower iris*

In regeneration of the lens from the upper iris, depigmentation occurs also in the lower iris. Fischel considers this change as participation of the lower iris in the first stage of regeneration. But true regeneration never takes place from the lower iris. Fischel ('00, '03) demonstrated one such case and maintained his interpretation against Wolff's objection, but his case is not without doubt, because the small lentoid, which is in connection with the lower iris and also in contact with the larger regularly formed lens, might have arisen from the latter and only secondarily come into connection with the lower iris edge.

Now the question naturally arises whether regeneration may occur from the lower iris, if it is prevented from occurring from the upper iris. For this study, total synechia of the upper iris was brought about in the following way. The cornea was cut horizontally in the upper part. After removal of the lens through

this wound, the upper iris was also drawn out and kept in place. All animals, ten in number, were killed in from fifteen to twenty-two days after operation. In case of successful operation, the anterior surface as well as the edge of the upper iris fused with the cornea. In four cases synechia did not occur and the lens vesicle started to regenerate from the edge more or less normally. In the other five specimens no typical regeneration took place from the upper iris, which fused tightly by its entire surface with the cornea, though the iris edge itself was rather free in these cases. The reason for the absence of regeneration from the upper iris edge in spite of its relative freedom is hard to ascertain, but insufficient blood supply due to fusion might account for it.

In all these five cases the lower iris presented no indication of regeneration at all, except the usual slight changes. Thus it was established that the lower iris is incapable of producing a lens even when regeneration from the upper iris is prevented.

*Regeneration after making a flap from the upper iris*

It is not without interest to find out where regeneration will start when a flap is made from the upper iris. The iris is cut above the blood vessel which encircles the pupil, together with the cornea. After being stretched by pressure of the lens the iris is then cut at one end and the lens extracted. The flap is then pushed toward the middle of the pupil to prevent fusion. This operation was done upon forty-five specimens. In a number of cases regeneration occurred quite normally, probably owing to the healing of the flap with the original iris. In others the flap disappeared and the lens was formed from the shortened iris, while some cases presented abnormalities as results of operation. Description of the latter cases is given here.

A. Twelve days after operation. The stump of the flap fused with the lower iris edge, dividing the pupil into two parts. At the fused part the flap is thick and irregular in its cell arrangement and some of these cells show distinct depigmentation, but there is no sign of regular regeneration. Depigmentation is not seen at the lower iris. The upper iris is thick and the pigment-free part



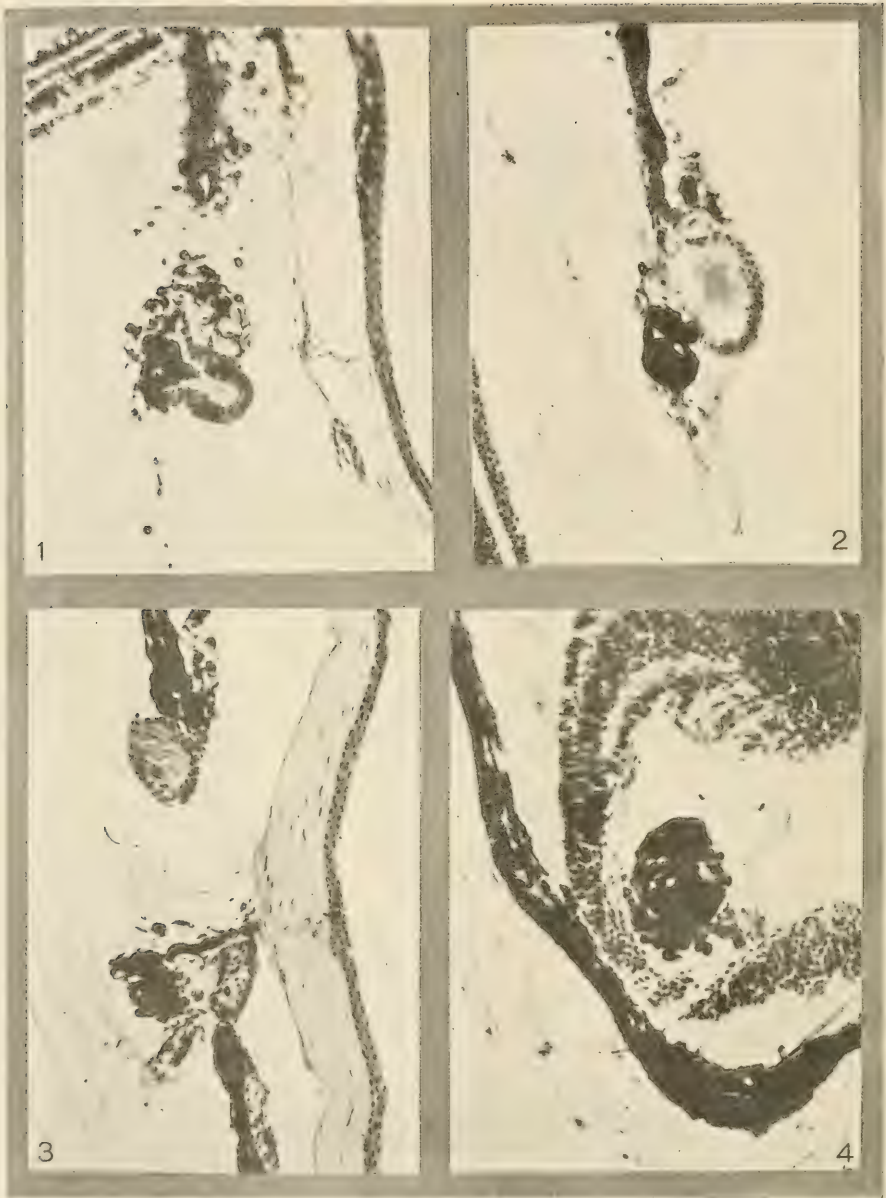


Fig. 1 Exp. C. Regeneration of the lens from an iris flap. The lens vesicle is formed anteriorly. No sign of regeneration at the shortened iris edge.  $\times 80$ .

Fig. 2 Exp. D. Regeneration of the lens from the cut edge of the iris. The lens vesicle is formed from the cut edge, while the iris flap remains unchanged.  $\times 80$ .

Fig. 3 Exp. A. Regeneration of the lens from a piece of the iris transplanted to the chamber.  $\times 80$ .

Fig. 4 Exp. C. Regeneration of the lens from a piece of the iris transplanted to the retina.  $\times 80$ .

of the inner layer is rather limited in its extent. Thus the pupil has in its upper margin the cut and the intact edge of the iris. The lens vesicle regenerated from the intact side and not from the cut edge.

B. Thirteen days after operation. In this case also the iris flap fused with the lower iris edge; but, in contrast to the former case, regeneration took place both from the cut iris edges as well as from the flap, the latter being converted totally into an incomplete lens vesicle.

C. Fourteen days after operation (fig. 1). The stump of the iris flap fused with the original upper iris, while the middle part of the flap is free from it. Almost the whole flap is changing into a lens vesicle, which turns toward the cornea. The cells enclosing the vesicle are columnar, pigment-free, and arranged partly in two layers. The cavity contains pigment cells and clusters. It can be easily concluded that the vesicle is the flap itself, because the iris stroma is attached to its anterior side. Although the cut edge of the original iris is quite free and two epithelial layers are fused together, no indication of true regeneration, such as thickening and depigmentation of the epithelium, can be seen, except that there is slight decrease of pigmentation.

D. Twenty-eight days after operation (fig. 2). In this case the flap remained unchanged and lens regeneration started from the cut edge of the iris. A microscopical study reveals a well developed lens vesicle between the original iris and the flap, which is closely attached to the vesicle and gives no depigmentation or other sign of regeneration. As the lens vesicle is pretty well developed, there is no connection with the cut edge of the iris, though in the inner layer distinct depigmentation is visible. This vesicle was formed undoubtedly from the upper iris.

As the results are different in the individual cases, no general conclusion can be drawn from this experiment.

*Effect of turning up of the upper iris edge*

When the lens regenerates from the iris edge, the lens fibers are formed always from the posterior wall of the lens vesicle. This led Wachs to assume that the direction of the lens fibers is determined by the presence of the retina. To examine how far this assumption is true, the iris edge was intentionally turned up, in such a way that the upper iris was cut at both sides and turned up in front of or behind the iris. Fifteen specimens were operated upon. In many cases, the bent iris did not remain in place, but came back to the original position, starting normal lens regeneration. Five cases disclosed some variations.

A. Thirteen days after operation. The iris has been only partially turned backwards, the lens vesicle accordingly was partially turned backward.

B. Sixteen days after operation. The lens vesicle regenerated from the iris edge posteriorly and slightly dorsally, without taking the normal downward direction, so that the lens fibers run as a whole from above downward postero-anteriorly.

C. Eighteen days after operation. The iris had been turned up anteriorly. No typical regeneration was obtained except slight depigmentation and grouping of cells at the edge.

D. Twenty-one days after operation. The iris was shortened. From the edge a relatively flat lens was formed. The lens fibers run as a whole slightly from above downward postero-anteriorly.

E. Twenty-three days after operation. Double lenses were formed. One is normal; the other quite abnormal and projects into the anterior chamber from the upper part of the former, the two lenses being partially continuous. A piece of the iris is attached behind the lens. The iris may have been severed by the operation in this case.

If the retina alone determines the direction of the lens fibers, the latter, whatever position the lens may take, must maintain as a whole a sagittal direction, because the retinal influence should remain the same. However, in the above cases the lens fibers

take sometimes a slightly downward direction. I am inclined to think that some factor influencing the direction of the lens fibers might also exist in the iris itself, because the direction of the axis of the lens seems to be vertical to the stalk of the lens, even in abnormal position.

*Regeneration from transplanted pieces of iris*

Fischel and Wachs studied cases where the lens regenerated from isolated or transplanted pieces of iris. In fourteen larvae Wachs cut the piece from the upper iris after extraction of the lens and left it in the posterior chamber. He found after some time that in six cases the pieces had slipped out, in one case it had remained in the corneal wound, in six cases they had fused with the iris, and in one case a lens was formed from the isolated piece, though incompletely. He transplanted also pieces of iris taken from other animals into the eyes of three specimens, but in all cases the pieces fused with the iris and then started to form lenses.

In fifteen specimens I made transplantations of upper iris pieces, taken from other individuals, into eye cavities. Examination after from thirteen to thirty-three days revealed that the pieces had remained intact in twelve cases; in nine cases they were in contact with the corneal wounds and in three cases they were apart from the cornea. The remaining three cases presented no trace of the transplanted pieces. None of these twelve cases gave pictures of regeneration, except that there was distinct depigmentation in one case.

Then I tried to transplant the upper iris pieces to the retina. Twelve specimens were operated upon and examined after from nineteen to twenty-six days. The results were as follows: In three cases the iris pieces were absent in nine cases the transplantation was successful. Among the nine successful cases, five showed regeneration (four in the retina and one in the anterior chamber), and four cases showed no regeneration.

Description of the regenerated cases follows:



A. Nineteen days after operation (fig. 3). What was unsuccessful in the former experiment, was achieved in this case. The transplanted iris piece is located in the anterior chamber near the lower iris edge, instead of being attached to the retina. The lens vesicle was formed anteriorly from the piece. The vesicle is elliptical. The cells are pigment-free, partly cuboidal, partly columnar, and arranged regularly in a simple layer. It is connected by a stalk with the iris piece. From the upper iris there is regular lens regeneration.

B. Twenty-two days after operation. The transplanted iris piece rests upon the lower part of the retina, which consists here of only two layers (inner reticular and outer nuclear). This is regenerated retina. The piece presents a round cell mass, amidst which there is a small vesicle, surrounded by a single layer of cuboidal pigment-free cells. Mitosis is seen in these cells. This structure must be considered as an early stage of a regenerating lens vesicle. It is somewhat striking that the vesicle was formed amidst the cell mass of the piece, while it usually starts from the periphery.

C. Twenty-two days after operation (fig. 4). The iris piece, resting on the retina, forms a round cell mass, measuring  $17\ \mu$  in diameter. In the center a cavity is formed, which is partly filled with cells continuous with the wall. The wall consists of one or two layers of cuboidal or columnar cells. Some of them are pigment-free and show mitosis. This also should be considered as a lens vesicle.

D. Twenty-four days after operation. The iris piece, transplanted into the retina, consists of two parts, pigment-containing and pigment-free. In the latter there is a vesicle consisting of cuboidal or columnar pigment-free cells. The cavity of the vesicle is small. Although this is imperfect, it also can be considered as a lens vesicle.

From the above it is seen that when pieces of iris are transplanted to the retina, they regenerate far better than in the eye cavity. Of course in all these cases regeneration remains, as might be expected, incomplete and does not attain even the stage of formation of lens fibers, but the structures formed are

recognizable as lenses by their depigmentation, their cell arrangement and their general form.

The fact that regeneration takes place more readily after transplantation to the retina seems to be explicable in one of two ways: 1) The iris piece is better nourished in the retina than in the eye cavity or, 2) the retina has special ability to induce the regeneration from the iris. This agrees with the assumption made by Wachs. At present it cannot be decided which possibility is more probable.

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Resumen por el autor, C. M. Child,  
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Estudios sobre la dinámica de la morfogénesis y herencia en la  
reproducción experimental.

## XI. Factores fisiológicos en el desarrollo de la cabeza de la planaria.

Las diferentes formas de cabeza observadas hasta el presente en la reconstrucción de trozos de *Planaria dorotocephala* pueden distribuirse en dos grupos principales. Esta serie, que comprende diversas condiciones desde la normal hasta la acéfala, representa una serie de inhibiciones diferenciales del desarrollo, siendo la inhibición mayor en la región media, decreciendo lateralmente. Bajo ciertas condiciones pueden tener lugar cambios secundarios en la forma de la cabeza, en dirección opuesta a las inhibiciones diferenciales. Estos cambios son el resultado de la aclimatación diferencial a la acción del factor inhibidor o de la regeneración diferencial después de su acción. El desarrollo de todos los términos de esta serie puede influirse experimentalmente mediante la acción de factores externos físicos y químicos y de factores fisiológicos internos. Las diferentes formas de cabeza están determinadas no por la acción específica de los agentes particulares o las condiciones del protoplasma, sino por el cambio de la condición fisiológica del protoplasma, la cual es fundamentalmente cuantitativa. La acción regional diferencial de los agentes físicos y químicos es el resultado de la presencia de gradientes fisiológicos en el protoplasma. Faltando dichos gradientes, una serie gradual de formas de cabeza diferencialmente inhibidas, desde la forma normal hasta la acéfala, sería imposible. La presencia o ausencia de órganos particulares en estas cabezas, como por ejemplo, los ojos, no supone la presencia o ausencia de los factores hereditarios o de las potencialidades correspondientes, sino simplemente la existencia de diferencias, primordialmente cuantitativas, en las condiciones necesarias para la realización de las potencialidades hereditarias.

## STUDIES ON THE DYNAMICS OF MORPHOGENESIS AND INHERITANCE IN EXPERIMENTAL REPRODUCTION

### XI. PHYSIOLOGICAL FACTORS IN THE DEVELOPMENT OF THE PLANARIAN HEAD

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THIRTY-THREE FIGURES

Earlier papers of this series and several other papers have dealt with various aspects of the reconstititional development of *Planaria dorotocephala* in relation to experimental and physiological conditions. It has been shown that development may be altered and controlled within very wide limits (Child, '09, '10 a, b, '11 a, b, c, '14 b, '16 a, '20 a) and that widely different developmental results, ranging from normal development to complete absence of certain organs or organ complexes and including different localizations and proportions of parts and organs, may be determined and controlled by physiological changes determined in a quantitative, rather than a specific way (Behre, '18, Child, '11 a, '14 b, '16, '20 a). The head has proved be of particular interest in these experiments, because of the possibility of experimental control and modification of the development of this most highly specialized region of the body with the production of a graded series of head-forms ranging from the normal to complete absence.

Experiments of various investigators have shown that very small pieces of the planarian body may give rise to heads alone. These facts demonstrate that the head is able to develop in the complete absence of other parts. This is true not only for *Planaria*, but for other relatively simple organisms in which re-



constitutional development of a head or apical end occurs (Child, '15 b, chap. IX). Moreover, the head is the chief organ of physiological dominance or control, in short of physiological integration (Child, '10 b, '11 c, d, 12, '15 b, chap. IX), it represents the high end of the chief physiological gradient in the body (Child, '13 b) and finally the graded series of head-forms, ranging from normal to complete absence, may be determined and controlled by many different agents and conditions, both external and physiological. The present paper is primarily concerned with the problem of head-development in its relations, on the one hand, to these experimental and physiological conditions and, on the other, to the hereditary constitution of the protoplasm.

#### HEAD-FORM AND HEAD-FREQUENCY IN RELATION TO EXTERNAL AND INTERNAL CONDITIONS

It has been pointed out in various papers (Child, '11 a, b, '15 b, pp. 105—108) that a series of head-forms exists in the reconstitution of pieces of *Planaria dorotocephala*. The terms of this series have been separated for convenience into the groups: normal, teratophthalmic, teratomorphic, anophthalmic, and acephalic. These different forms of head, briefly described in earlier papers and considered more fully below, evidently represent various degrees of inhibition of head-development, ranging from the very slight degree of inhibition in the teratophthalmic to the complete inhibition of the acephalic form. It has also been shown that in material which is physiologically standardized as far as possible as regards physiological age or size and nutritive condition, pieces with anterior ends at a given level of the body show a decrease in head-frequency with decrease in length of the piece (Child, '11 a, b, d). Second, comparing pieces of the same length from different levels of the body, we find that head-frequency is highest at levels nearest the original head and decreases as the level of the cut surface becomes more and more posterior, back to the level of fission, where the head-frequency suddenly increases again (Child, '11 b, d). Posterior to the usual level of fission the different zooids are physiologically

more or less clearly indicated by the differences in head-frequency at different levels (Child, '11 d). In pieces representing the same fraction of body-length the head-frequency is lower in smaller, physiologically younger animals than in larger, older animals and lower in starved than in well-fed animals (Child, '11a, '20 a). In pieces of like size from animals of the same size and in the same physiological condition, the head-frequency is higher in pieces excited to active locomotion during the early stages of reconstitution than in pieces remaining undisturbed (Child, '11a, '20 a).

On the other hand, it has been possible to control and alter head-frequency by means of various external physicochemical factors. KNC, for example, decreases head-frequency at levels near the head and, except in relatively high concentrations, increases it at levels near the posterior end of the first zooid and produces little change at intermediate levels (Child, '16 a). Extensive work by Mr. J.W. Buchanan, which is not yet published, shows that various anesthetics act in essentially the same way as cyanides on head-frequency. Some work not yet completed, with agents which act chiefly as accelerants rather than inhibitors indicates that such agents alter head-frequency in the opposite direction from the inhibiting agents. And finally, experiments with different temperatures have shown that both high and low temperature are very effective in altering head-frequency (Behre, '18). Most of these experiments have been used in laboratory class work and have therefore been repeated by many different persons, with essentially identical results. In general the inhibiting agents decrease head-frequency in pieces from regions near the head, where it is normally highest, and increase it in pieces near the posterior end of the first zooid, where it is normally lowest. It has been shown by increase in susceptibility and in  $\text{CO}_2$  production that the pieces are temporarily stimulated by the act of section (Child, '14 a, Robbins and Child, '20), and data on oxygen consumption obtained by Doctor Hyman and soon to be published also demonstrate this stimulation. The data on stimulation show that in pieces of given length, the stimulation is least at levels nearest the

head and greatest at levels nearest the posterior end of the first zooid, pieces from intermediate levels showing intermediate degrees of stimulation (Child, '14 b) and that in pieces of different length with anterior ends at the same level, the shorter pieces are more stimulated than the longer. And finally, it has been shown that it is determined during this period of stimulation following section whether or not a piece shall give rise to a head (Child, '14 b), and that the greater the degree of stimulation, the lower the head-frequency (Child, '14 a, b). According to the experimental data, then, this stimulation of the pieces following section inhibits rather than favors head-development. As pointed out elsewhere, this relation between head-frequency and stimulation of piece enables us to understand how the same concentration of an inhibiting agent such as KNC may alter head-frequency in two opposite directions. First, it may inhibit to a greater or less extent the activity of the cells directly concerned in head-development and so decrease head-frequency. Second, it may inhibit or decrease the stimulation following section, i.e., it may inhibit the physiological factor which inhibits head-formation and so may actually increase head-frequency under certain conditions (Child, '16 a). In the same paper it was shown that relatively short exposures to the agent, which merely prevent the temporary stimulation and permit recovery of the head-forming cells are more effective in increasing head-frequency, while exposures continuing through a considerable part of the head-development are more effective in decreasing head-frequency. This is of course to be expected from the facts.

The data of experiment in general force us to the conclusion that head-development is not determined by other regions of the piece, though it may be inhibited by them. Head-development is a self-differentiation which occurs if the cells near the cut surface undergo a sufficient degree of dedifferentiation and attain a sufficiently high rate of metabolism. In pieces undergoing reconstitution, whatever the level of the pieces the head arises directly from the cut surface, and it is evident that in all cases except at levels directly behind the original head, the



new head is out of place and develops at the expense of whatever regions of the body adjoin the cut surface. In fact, it may be said to develop in spite of other parts of the piece, and the more intense the stimulation of these parts, the less rapid and complete is the dedifferentiation of the cells at the cut surface and therefore the greater the degree of inhibition of head-development.

Under all conditions thus far analyzed, whether these are internal, such as region of body, degree of stimulation, nutritive condition, and physiological age, or external physicochemical conditions, the series of head-forms from normal to acephalic is essentially the same. In other words, there is no evidence to indicate any specific relation between particular head-forms and particular conditions or agents. Slight minor differences with different agents may indicate specificities, but it is perfectly evident that the series of head-forms in general represents quantitative gradations rather than specific differences in developmental factors.

#### DIFFERENTIAL SUSCEPTIBILITY

It has been demonstrated for several hundred species of organisms, including the chief animal groups as well as many plants, that from the earliest embryonic stages on, definite and characteristic differences in susceptibility are related in a definite way to the physiological axes (Child, '20 b, pp. 154-162, and references there given). Moreover, it has been found that in general among the simpler organisms and in the earlier stages of development susceptibility to a certain range of relatively high concentrations or intensities of at least many external agents varies directly, though not necessarily proportionally, with the rate of certain fundamental physiological activities and that the ability to become acclimated to certain lower ranges of concentration and to recover from temporary exposure varies in the same way. This relation between susceptibility and physiological condition is fundamentally quantitative in character. In the more highly differentiated stages and organisms this simple relation is altered and complicated by various, ap-

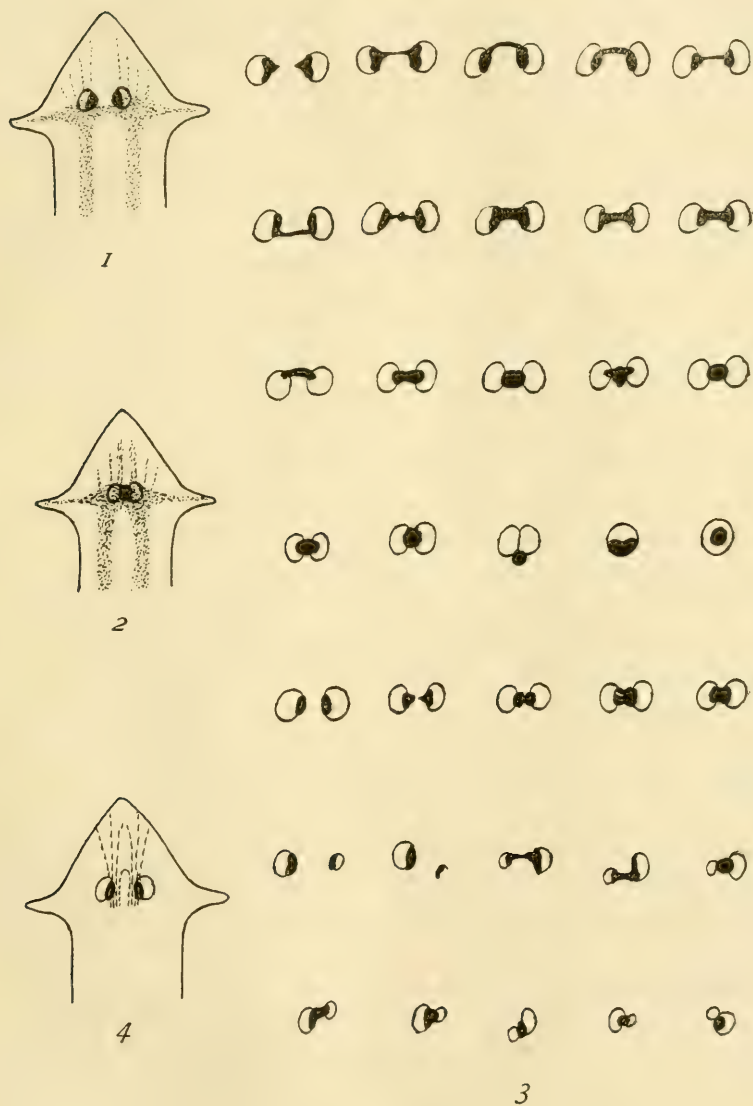


parently specific relations between particular organs or tissues and particular agents. Many different lines of evidence indicate that the physiological axes are primarily quantitative gradients in physiological condition and metabolic rate and that the differential susceptibility at different levels of an axis results from this difference (Child, '20 b). The region from which the head, or apical end develops, being primarily the most active region, is most susceptible to higher and most capable of acclimation to lower concentrations and intensities of external agents. It has been demonstrated in various ways that this relation holds good for the head of *Planaria* (Child, '11 c, '12, '13 a, b, '16 a).

It has also been shown that differential susceptibility, besides determining different survival times of different regions, determines developmental modifications in two opposite directions, differential inhibition on the one hand and differential acclimation or recovery and in some cases differential acceleration on the other (Child, '16 b, '17; Bellamy, '19). The graded series of head-forms in *Planaria* is evidently the result of a non-specific action, since all terms of the series are produced by many different external and internal conditions. Moreover, since different regions of the head are affected in different degree in the different forms, a differential action of the determining factor or factors is evidently concerned. It remains, then, to be determined whether, or to what extent, the series of head-forms can be interpreted in terms of differential susceptibility.

#### DIFFERENTIAL INHIBITION IN HEAD-DEVELOPMENT

*The normal head.* The normal head of *Planaria dorotocephala* (fig. 1) possesses certain rather definite characteristics as regards external form localization of sense organs and position, form and structure of the cephalic ganglia. The ganglionic mass, unquestionably the primary organ of the head, is in the normal head, distinctly double, consisting of right and left halves, connected by a commissure. The same bilaterality is also evident in the localization and innervation of the sense organs



Figures 1 to 4

and in the form of the head. Obviously, the normal head represents a certain proportionality of parts which in some way is determined as the usual result of development.

*The teratophthalmic head.* The shape of the head is normal, but the eyes show more or less approximation to the median line, and section shows that the ganglia are also more or less approximated with reduction or complete absence of the commissure between them (fig. 2; also Child and McKie, '11). Figure 3 shows some of the eye-forms characteristic of the teratophthalmic head, the first five horizontal rows of the figure representing bilaterally symmetrical, the last two rows asymmetrical forms. The first four rows show forms in which more or less continuity of pigment appears, even when the eyes are a considerable distance apart. In the fifth row are shown cases in which the pigment cups remain distinct until the eyes are rather closely approximated. The order in each series follows in general the degree of departure from the normal eye-form. The asymmetry of the series in the last two rows of figure 3 is the result of purely incidental factors which determine a difference in rate of development on the two sides of the median line. For example, obliquity of cut surface may determine temporary asymmetry, the more posterior levels developing more slowly because they represent lower levels of the longitudinal gradient. Again, if the amount of entodermal tissue exposed at the cut surface is much greater on one side than on the other, the exposure of parenchyma is less and the rate of head-development is slower on the side with the greater entodermal exposure. The asymmetries of the eyes in this series are merely indications of the ganglionic asymmetries. In many cases both disappear in later development.

It is evident that the teratophthalmic head differs from the normal in the reduction or complete inhibition of the median region of the head, at least at the level of the ganglia. The degree of inhibition may range from the just appreciable approximation of eyes and ganglia to the complete inhibition of development of the region lying between the dotted lines in figure 4. Whether the inhibition of the median region extends to the tip of the head

cannot be determined with certainty because of the absence of well-defined landmarks in this region, but the conditions found in the teratomorphic heads make it probable that at least in the more extreme teratophthalmic forms the median region is more or less inhibited throughout the whole length of the head.

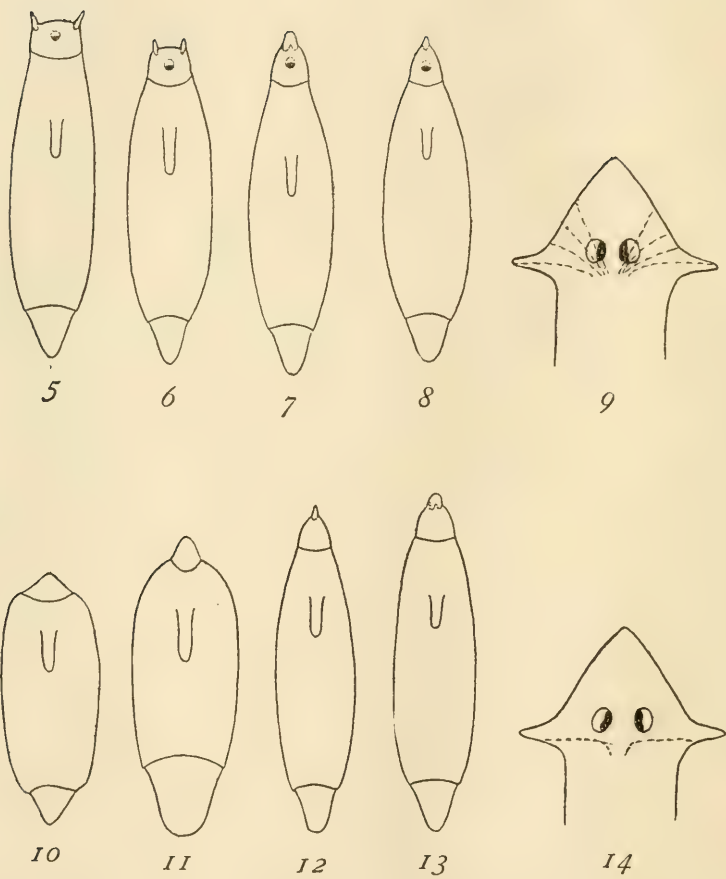
The series of dotted lines in figure 4 are intended to represent in a diagrammatic way approximately the regions inhibited in various degrees of teratophthalmia. At the level of the ganglia the degree of median inhibition can of course be determined with relative exactness by the degree of approximation of the eyes and in section of the ganglia. As regards the preganglionic region, however, the lines in figure 4 are merely suggestive.

Since the teratophthalmic head-form is determined by external and physiological conditions which retard or inhibit head-development to a greater or less degree, it is obviously a result of differential inhibition, the median region of the head being more susceptible and therefore more inhibited than the lateral regions.

*The teratomorphic head.* This is merely a more extreme degree of differential inhibition. This head-form usually shows a single median eye (figs. 5 to 8) and a ganglionic mass retaining only slight traces of the double structure of the normal ganglion (Child and McKie, '11). Here the differential inhibition evidently includes the median head region all the way to the tip, for this region is more or less completely absent, and therefore the cephalic lobes, lateral in normal heads, appear farther anteriorly and more or less approximated to the median line, until in the more extreme forms a single median cephalic lobe with double (fig. 7) or even single unpigmented sensory area at its base figure 8, develops. In these cases the whole triangular region of the head between the cephalic lobes has failed to develop. Figure 9 indicates diagrammatically the regions chiefly inhibited in the various degrees of teratomorphic development.

There is some overlapping as regards the eyes between teratophthalmic and teratomorphic forms. Occasionally in heads distinctly teratomorphic as regards other features, more or less distinctly double, instead of single eyes appear, and heads which





Figures 5 to 14

are normal as regards position of cephalic lobes and preganglionic development show only a single eye.

*The anophthalmic head.* This represents a still more extreme stage of differential inhibition, the eyes and usually the cephalic lobes being absent and the ganglionic mass remaining rudimentary. Externally the anophthalmic head usually remains without distinguishable organs of special sense (figs. 10, 11), though sometimes a partially double or wholly single cephalic lobe appears at the tip (figs. 12, 13). Except in some cases of very slight development of new tissue, behavior of anophthalmic animals differs distinctly from that of acephalic forms, being more like that of forms with more highly developed heads, and section shows at least a rudimentary ganglion. As indicated in figure 14, the anophthalmic head represents almost complete inhibition of head development.

*The acephalic form.* Here development of a head is completely inhibited, the cut surface contracts, the wound is merely filled in with cells, and healing occurs without outgrowth (figs. 15, 16). Even here, however, differences in the contraction of cut surface and amount of new tissue appear in different headless pieces, as figures 15 and 16 indicate. Such differences are of course dependent on the degree or completeness of inhibition of outgrowth.

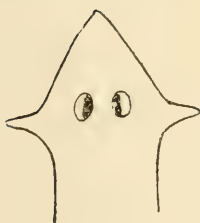
This series of head-forms from normal to acephalic evidently represents different degrees of differential inhibition. The differential between median and lateral is clearly marked, the median region being most susceptible and so most inhibited and susceptibility decreasing laterally. The longitudinal differential also appears to some extent, the ganglionic region being the most susceptible region, but the difference between it and the preganglionic region is slight. In consequence of these susceptibility differentials, inhibition of head-development involves first the median ganglionic region and then progressively the anterior and lateral regions, until head-development is completely inhibited. It must be remembered that the differential susceptibility determining the different head-forms is that of the earlier stages of head-development, and that the susceptibility relations during



15



16



17



18



19



20



21

Figures 15 to 21

stages are not wholly identical with those of the fully developed head with its specialized sense organs. The facts indicate that the ganglionic region is primarily the region of highest susceptibility in the whole head and that the preganglionic region arises as a secondary gradient in the opposite direction from that of the rest of the body. Apparently similar conditions exist during development in at least many other animal groups, even the vertebrates, the most susceptible region during all except perhaps the earliest stage being not the extreme anterior tip of the head, but a somewhat posterior level. Such secondary gradients arise during the course of development and may even disappear later, but it is difficult to determine with certainty how far later changes in susceptibility to particular agents are due to quantitative differences in physiological condition and how far to specific relations. Only when it has been found that, as in earlier stages, the susceptibility relations are essentially the same for a given range of concentration or intensity of many different agents is it reasonably certain that the relations are primarily quantitative rather than specific.

#### DIFFERENTIAL ACCLIMATION AND RECOVERY IN HEAD DEVELOPMENT

Under conditions which permit acclimation or recovery to occur, we find indications of differential effect similar to those already observed in other forms (Child, '16 b, '17; Bellamy, '19); that is, the changes are in the opposite direction from those involved in differential inhibition. Some of these changes observed are briefly described.

*Differential acclimation in normal heads.* When intact animals with normal heads (fig. 17) are placed in low concentrations of various anesthetics, e.g., ethyl alcohol 1.25 to 1.5 per cent; ethyl ether 0.2 to 0.4 per cent, the solutions being renewed every two two days or oftener, the whole head decreases in size relatively to the body, but the preganglionic region undergoes more or less complete reduction, either by resorption or disintegration, until after sixteen to twenty days the head resembles figure 18. About this time, however, or soon after, new growth begins in the median



portion of the preganglionic region, in the same solution which brought about the earlier reduction of this region, and ten to fourteen days later, that is, a month after the animals were first placed in the solution, the heads range in form between figures 19 and 20. The whole head has undergone increase in size, but the median region has grown much more rapidly than any other part and has given rise to a disproportionately large preganglionic region. This region is functional and is bent and twisted about in locomotion: it very evidently represents merely an overdevelopment of the median preganglionic region with its sensory margins. Its development can mean nothing else than that the cells of the median region become, after a certain length of time in the solution, more capable of growth than those of the more lateral regions and that under these conditions the median region grows to larger than normal size because its ability to use nutritive material and to synthesize new protoplasm is relatively greater as compared with that of lateral regions, though actually of course less than in the normal animal. In other words, the alteration of size and proportion means an alteration in the metabolic relations of median and lateral regions in consequence of differences in their susceptibility and capacity for acclimation.

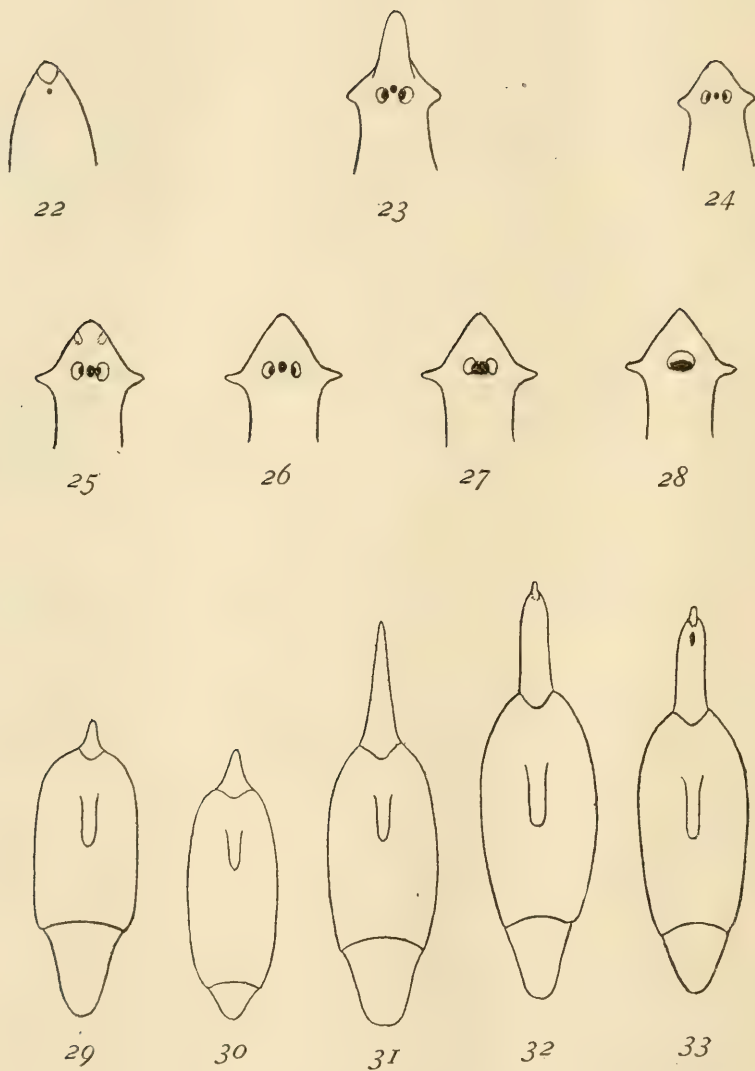
Animals with heads like figures 19 and 20, when removed from the anesthetic solution to water, show a gradual approach to the normal head-form. After two months in water some heads may be nearly normal, but the preganglionic region is usually still somewhat larger than normal. It is probable that completely normal proportions would be reached in time, but none of these experiments was continued longer than two months after return to water.

*Differential acclimation in development of new heads.* When pieces undergo reconstitution in alcohol (1.25 to 1.5 per cent) or ether (0.2 to 0.4 per cent) or in low concentrations of chloretone or of various other anesthetics, the development of the head is at first markedly inhibited. A very common form of head under such conditions is that shown in figure 22. Here the outgrowth of new tissue at the cut surface is largely inhibited, but rediffer-

entiation has occurred behind this surface and a single median eye has developed. If differentiation proceeded far enough, such a head would undoubtedly be teratomorphic. This form of head develops in a week or ten days, but after two weeks or more, even though kept in the anesthetic, further development usually occurs, resulting in a form with overdevelopment of the median region like figure 23, or sometimes in a head of normal or nearly normal form (fig. 24). Figure 23 shows the same type of head as figures 19 and 20. This head-form is, in fact, characteristic of conditions which inhibit head-development at first, but permit some degree of acclimation.

Figures 23 and 24 show another interesting feature. In the earlier stages these heads possess a single median eye, but when acclimation and further development take place, two more eyes appear in normal position, so that the head finally possesses three eyes. This must mean that in the earlier development the median head region is so far inhibited that conditions determining differentiation of eyes exist only at the median line. The region between the eyes of normal heads is completely inhibited. Later, when acclimation occurs, the median regions develop further and eyes and nerves develop in normal relations. The less inhibited and more completely acclimated heads approach normal form, except as regards the median eye (fig. 24), while less complete acclimation gives forms like figure 23. These latter, however, if returned to water, gradually approach normal proportions.

*Secondary transformation of teratomorphic heads.* The teratomorphic head apparently represents a rather unstable equilibrium, for its frequency is usually much lower than that of other head-forms, and heads which at first develop as teratomorphic not uncommonly undergo transformation after a week or two into teratophthalmic forms. This transformation may occur in water at room temperature, as well as in cases of acclimation to inhibiting external conditions. Figures 25 to 28 show heads which were originally teratomorphic heads resembling those of figures 5 and 6. The transformation involves changes in both the cephalic lobes and the eyes, besides the internal



Figures 22 to 33

changes. The original cephalic lobes are resorbed or atrophy and new ones develop in normal position. In figure 25 the old disappearing lobes are indicated near the tip of the head. In the stage figured here the lobes as actual outgrowths have already disappeared, the two areas indicated in the figure representing the unpigmented sensory areas at the base of each lobe. The eye changes consist either in the development of two complete eyes in addition to the median eye (figs. 25, 26) or in various degrees of teratophthalmia (figs. 27, 28), and the median regions of the ganglia also develop more or less completely. Such transformation has been observed repeatedly in all its stages. It occurs chiefly in the less extreme teratomorphic types, such as figures 5 and 6, the more extreme types (figs. 7, 8) being in most cases permanent.

As pointed out above, the teratomorphic head results from inhibition of the median regions to a certain degree, and it is evident that the secondary transformation of such heads represents more or less complete acclimation to, or recovery from, the earlier differential inhibition; that is, the median regions at first inhibited, later undergo more or less complete development.

*Secondary transformation of anophthalmic heads.* Heads which develop at first as anophthalmic heads of ordinary form (figs 10, 11) very often show secondary elongation, particularly of the median region (figs. 29 to 31), and in some cases a median cephalic lobe develops at the tip (fig. 32) and occasionally after two or three weeks a single median eye (fig. 33); i.e., the anophthalmic may in some cases become secondarily a teratomorphic head. Here again it is evident that the secondary transformation results from growth and often hypertrophy of those head regions which were at first most inhibited.

*Secondary transformation of acephalic forms.* Such transformation is also of very frequent occurrence in acclimation to, or recovery from, inhibiting factors. Pieces which remain acephalic indefinitely under the inhibiting conditions may develop anophthalmic, or in some cases teratomorphic, and occasionally even teratophthalmic heads when acclimation or recovery occurs. Acclimation usually results in forms like



figures 29 to 33, while in recovery after removal of the inhibiting factor the more nearly normal head-forms may appear. In temperature experiments, for example, pieces which remain acephalic for several weeks at low temperature ( $3^{\circ}$  to  $5^{\circ}\text{C}.$ ) sometimes begin to develop anophthalmic or teratomorphic heads after this time, even when kept at the low temperature, but if removed to  $20^{\circ}\text{C}.$  many develop teratophthalmic and some even normal heads.

#### DIFFERENTIAL SUSCEPTIBILITY IN DEVELOPMENT IN GENERAL

It will be noted that in the heads of *Planaria*, as well as in the development of the sea-urchin (Child '16 b), the polychete (Child, '17) and the frog (Bellamy, '19) departures from the normal occur under the experimental conditions employed in two opposite directions. In the differential inhibitions certain regions are affected to a greater degree than others, and the parts to which the regions most affected normally give rise are more or less reduced or completely absent. In the differential acclimations and recoveries the parts originally most inhibited develop more or less completely or may even show over-development. The most extreme types of differential acclimation (figs. 19, 20, 23) have thus far been observed only after the use of external agents which produce at first marked inhibition, but in which a considerable degree of acclimation occurs. The habit-forming inhibiting drugs, for example, so far as they have been used, are particularly favorable agents for the appearance of these secondary changes. In cyanide, on the other hand, in which acclimation is very slight and occurs very slowly, the more extreme acclimation forms have never been seen. All other head-forms except these extreme acclimation forms, not only the differential inhibition series normal to acephalic, but the less extreme acclimation forms, can be and have been determined by all of the external chemical and physical agents used in my experiments, as well as by the physiological conditions associated with differences in size of piece, region of body, nutrition, age, etc. These head-forms are, in short, the morphological effects of physiological

changes which are non-specific; that is, quantitative in character. The same is true of the differential inhibition and acclimation forms in sea-urchins and polychetes. Moreover, even in the vertebrates similar developmental modifications may be similarly produced. In the frog, for example, Bellamy has produced experimentally a series of differential modifications of development essentially similar to those in *Planaria* as regards axial relations. The differential inhibitions of head-development range from normal to almost acephalic, through a series of forms closely comparable to those in *Planaria* in that they all represent a greater degree of inhibition of median than of lateral regions. The differential acclimations and recoveries, on the other hand, represent changes in the opposite direction. Only some of the data on the earlier stages have as yet been published (Bellamy, '19). It is also evident that the cases of cyclopia experimentally produced by Stockard ('07, '09, '10, '11) in fishes are cases of differential inhibition similar to those in *Planaria* and other forms. In these cyclopias the median region of the head is more inhibited in early developmental stages than lateral regions. The intermediate forms between the complete cyclopias and the normal heads are closely comparable to the various degrees of differential inhibition in the planarian head. The occurrence of essentially similar developmental modifications in such widely different organisms and under so wide a range of experimental conditions indicates the existence in all these forms of certain non-specific or quantitative physiological factors concerned with the localization, growth, and differentiation of parts. These factors are, as so often pointed out, the physiological gradients. The median region of the head is, on the one hand, more inhibited than lateral regions and, on the other, acclimates or recovers more rapidly or more completely, because it represents the 'high' region of a physiological gradient; i.e., a region differing in its physiological state from other levels of the gradient in such manner that the rate of the fundamental metabolic reactions is higher in it than at other levels. Similar gradients of this sort may exist in protoplasts which are specifically very different, and their existence determines

the similarity of the differential modifications of development in different species and accounts for the fact that they represent a quantitative rather than a specific aspect of the action of physical and chemical agents upon protoplasms. In other words, these modifications result primarily from differences in rate, amount, intensity, or degree, etc., in protoplasm, rather than from differences in quality, but in so complex a physicochemical system as protoplasm qualitative differences are certainly frequently determined by quantitative changes. There can be no doubt, for example, that rate of fundamental metabolism in the cells which give rise to a new head in *Planaria* is one factor in determining whether or not eyes or cephalic lobes or even cephalic ganglia shall develop.

The significance of the physiological gradients has to do with these quantitative aspects of development. They represent merely quantitative physiological conditions in the protoplasm, which constitute factors in the realization of the hereditary potentialities of that particular protoplasm. The gradients create nothing; they do not determine the specific characteristics of particular organs; they merely determine whether a particular process of realization shall occur or where it shall be localized and how it shall proceed.

The fact that essentially the same developmental modifications may be produced by many different agents and conditions does not, of course, mean that all such agents and conditions act in exactly the same way upon protoplasm. It means merely that with certain ranges of intensity or concentration non-specific factors or quantitative factors in their action exist. It is these factors which constitute the basis of differential susceptibility. Undoubtedly different agents and conditions act in different ways upon the same protoplasm and the same agent or condition acts in different ways on different protoplasms, even upon different organs or tissues within the same individual. Notwithstanding these real or apparent specificities of relation between protoplasm and physicochemical agents, the non-specific aspects also exist, and in the simpler organisms and the earlier stages of development they play the larger part.



And finally, the fact must be emphasized that differential inhibitions, acclimations, and recoveries in development are dependent primarily upon the differences in susceptibility in the earlier developmental stages, rather than upon those observed in the fully developed animal. In *Planaria*, for example, as in other forms thus far examined, the data indicate very clearly that in the earlier stages of head-development the median region is more susceptible to higher and more able to acclimate to lower concentrations and intensities than lateral regions. In the fully developed normal head, however, the special sensory regions, e.g., the cephalic lobe and the preganglionic region are the most susceptible portions of the external surface (Child, '13b). Moreover, in the fully developed animal differences in the relative susceptibility appear with different agents (Child, '20 b, pp. 157-159). These real or apparent specificities are, however, of secondary origin and are associated with the progress of differentiation. Their existence does not in any way conflict with, or make impossible the existence of the general quantitative relations which determine the susceptibility gradients in the less highly specialized protoplasms.

#### INHERITANCE AND EXPERIMENTAL REPRODUCTION IN *PLANARIA*

It is sufficiently obvious that at least certain aspects of the problem of inheritance can be approached only through development; that is, knowledge of the hereditary potentialities of a particular protoplasm can be obtained only through the realization of these potentialities in development. Moreover, it is now generally recognized that so-called normal development represents only a certain range of realization among the hereditary potentialities. In other words, normal development is merely the particular complex and sequence of changes which is possible in a particular protoplasm under what we call normal conditions. The relatively high degree of uniformity in normal development results from the fact that it represents a process which is, so to speak, standardized by the relatively high degree of uniformity of the conditions under which it occurs.



When the conditions are altered, the actual behavior of the protoplasmic system is altered and other potentialities than the normal are realized.

As its title indicates, this series of papers has been regarded as a study of inheritance, as well as of development, because the facts of development established afford a basis for certain conclusions concerning inheritance. As regards the head of *Planaria*, for example, it is evident that all the different forms of head are just as truly inherited as the normal; that is, all of them represent potentialities of the physicochemical system which is the material substratum of inheritance. Which of these potentialities is realized depends upon the conditions of development. Or we may say if we wish that under certain external conditions of development a particular head-frequency is inherited for a certain size of piece from a certain body-region of animals of a certain size of physiological age and a certain nutritive condition. This, however, means exactly the same thing as the preceding form of statement.

If we attempt to retain a strictly preformistic conception of inheritance, the facts in the case of *Planaria* force us to the conclusion that a grouping or association of determinants, genes, or factors must exist for each form of head occurring in development, a particular grouping being activated by each particular complex of developmental conditions. But the head-forms constitute a graded series differing primarily in degree and an indefinite number of forms is possible. Any preformistic view which regards these groupings as actually existing in space and time in the germ plasm rather than as mere possibilities of action in a complex physicochemical system, leads either to absurdity or to some form of 'vitalism.'

The point to which I wish to call particular attention, however, is that developmental conditions which differ primarily in a quantitative rather than in a qualitative way may determine qualitative differences in the developmental realization of the hereditary potentialities. In other words, they may determine presence or absence of a particular organ or character. A certain degree of retardation or inhibition of development in *Planaria*

whether produced by low temperature or other external physical or chemical agents or by physiological conditions, will determine complete absence of eyes and of most or all of the ganglionic mass. The series of heads from normal to acephalic is physiologically a series whose terms differ primarily in a quantitative rather than a qualitative way, the qualitative differences which arise resulting from the quantitative differences. The same is true of any other graded series of forms resulting from differential susceptibility; e.g., the sea-urchin modifications (Child, '16 b). *Planaria* does not of course inherit any particular type of head, but merely the potentiality of giving rise under conditions differing in degree or quantity to a graded series of head-forms, with an indefinite number of terms in the series. The hereditary factors, genes, or whatever we prefer to call them which are concerned in head formation in *Planaria*, are unquestionably all present in all forms of head but the sort of head which appears is determined on the one hand by quantitative physiological differences between different regions of the body and on the other by primarily quantitative alteration of these conditions through the non-specific action of physical or chemical factors.

The physiological gradients of the whole body of *Planaria* or of any other axiate form are primarily quantitative, physiological feature, very similar in character in specifically different protoplasms, and such gradients are merely factors in determining the first steps of the process of realization of hereditary potentialities. A physiological gradient is merely a quantitative physiological factor affecting the action of the hereditary mechanism of a specific protoplasm. As pointed out elsewhere, it is the primary pattern of the organism (Child, '20 b). The protoplasm of the species with its hereditary constitution representing a wide range of potentialities is the material in which the pattern is, so to speak, worked out. The pattern, in its simplest terms the physiological gradient, is the organizing factor. The gradient determines localization, order, proportion, and even presence or absence of particular parts, but the specific characteristics of the parts present are determined by the hereditary constitution of the protoplasm.

This quantitative aspect of organization has been emphasized here because it is essential that we distinguish clearly between the specific hereditary mechanism or constitution of each particular protoplasm and the quantitative physiological factor which makes possible the development of a definite and orderly organism from this protoplasm. There has been much confusion on this point, both from the preformistic and the epigenetic view-point, but many different lines of evidence force us to the conclusion that, while the physicochemical constitution of organs or parts depends on the specific constitution of the protoplasm in which they develop, the existence of organization depends primarily upon graded quantitative differences in physiological condition in the protoplasm. Alteration of these quantitative factors determines alteration of the organization within the limits of the particular protoplasm. Under this category fall all the developmental modifications dependent upon differential susceptibility. They represent, in short, alterations of the organizing factor in a specific protoplasm. Such alterations according to the evidence, are initiated as quantitative changes, changes in degree, rate, intensity, rather than changes in kind, but the results, both morphological and physiological, may differ in kind, that is, qualitatively.

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### Estudios sobre los efectos de la sed.

## II. Los efectos de la sed sobre el crecimiento del cuerpo y de los diversos órganos de la rata albina joven.

En ratas albinas de un mes de edad, próximamente, se mantuvo constante su peso durante varias semanas alimentándolas con una pequeña cantidad de líquido (leche) que constituye una dieta adecuada para el crecimiento. Las ratas así tratadas presentan una tolerancia progresiva de la sed, de tal modo que cada día se necesita menos leche para mantenerlas durante el experimento. La cola crece en longitud pero la longitud del cuerpo permanece constante. El peso del esqueleto aumenta marcadamente, mientras que el peso del grupo visceral aumenta ligeramente. La musculatura permanece casi constante. Aparece una ligera disminución en el tegumento y una pérdida de peso marcada en el resto del organismo. De las vísceras individuales: (1) La hipófisis, globos oculares, riñones, suprarrenales, médula espinal, esqueleto, nervios ciáticos, páncreas, estómago e intestinos, hígado y útero aumentan de peso de un modo definido. (2) El corazón, cerebro y los pulmones permanecen sensiblemente con el mismo peso. (3) El timo, ovarios, glándulas parótida y submaxilar, bazo, testículos, epidídimos y tiroides experimentan una disminución de peso más o menos marcada. La tendencia hacia el crecimiento en los diversos órganos de las ratas jóvenes durante los experimentos, corresponden en general a los encontrados en ratas de la misma edad durante experimentos sobre los efectos de una dieta deficiente, aun cuando existen algunas excepciones (testículos y riñones). Del mismo modo los resultados de los experimentos sobre la sed (también los de inanición) presentan una semejanza general con los llevados a cabo en los adultos. Existen sin embargo ciertas diferencias según la edad y también según el tipo de inanición.

STUDIES ON THE EFFECTS OF THIRST

II. EFFECTS OF THIRST UPON THE GROWTH OF THE BODY AND  
OF THE VARIOUS ORGANS IN YOUNG ALBINO RATS

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It has been shown by various investigators that if young animals are underfed so as to prevent an increase in body weight, changes nevertheless occur in various parts of the body. Some organs remain nearly constant in weight, others lose, and still others show a remarkably persistent growth in spite of the underfeeding. Since for growth water is no less essential than food (as demonstrated for the human infant by Meyer, '13), it is of interest to determine whether similar changes occur in the organs of young animals in which growth is prevented by a restriction of the water in the diet. The present investigation was undertaken for this purpose.

The work was done in the Institute of Anatomy of the University of Minnesota. This opportunity is taken to express my indebtedness to Dr. C. M. Jackson, Director of the Department, for valuable aid and direction.

#### MATERIAL AND METHODS

The albino rat (*Mus norvegicus albinus*) was chosen as the most convenient form for experiment. Postnatal growth norms for the body and various organs are available for comparison (Donaldson, '15). The changes in the weights of the organs have also been studied in young rats during underfeeding (Jackson, '15 a; Stewart, '18, '19) and in adult rats on a dry diet (Kudo, '21).

The material used in the present experiment included fifty-six individuals in all. The forty-one survivors (from eight litters) are listed in table 1. In the first column the letter (K) indicates the series, the number preceding the decimal point designates the litter, and the number following is for the individual rat. The letter "m" signifies male; "f" female. Litters K5, K7, K8, K9, K10, K11, K12, K13, and K14 include, respectively, 4, 1, 5, 2, 8, 3, 5, 2, and 11 individuals. These include 17 males and 24 females, a total of 41. In addition 15 rats died during the test. They are excluded from the tables because the coagulated blood due to postmortem congestion might affect the weights of the organs.

In most cases the experiment began when the rats were about four weeks of age. Some of the experiments began at the age of three weeks (time of weaning), but most of these rats died.

Of the 41 rats listed, 11 were used for controls and the other 30 for the tests. The test rats were held at nearly constant body weight for varying periods as follows: for about 1 week (3 females); 2 weeks (2 males, 1 female); 3 weeks (4 males, 2 females); 4 weeks (2 males); 5 weeks (2 males, 2 females); 6 weeks (1 male, 1 female); 7 weeks (1 male, 3 females); 8 weeks (1 female); 9 weeks (1 male, 2 females); 11 weeks (1 female); and 13 weeks (1 female). Of the 30 test rats listed in table 1, one (K9.2 m.) of the 9-weeks' group was omitted, on account of its large body weight, from the corresponding group in table 2.

Eight normal controls (3 males, 5 females) were killed at the beginning of the experiment. The additional normal controls (K5.2, K 5.3, K 8.5) were killed at the end of the time periods indicated. These, and also two of the other controls (K7.1 and K9.1) are not used in table 2, because their body weights are too great for direct comparison with the test rats. A large number of observations upon normal rats previously published by Donaldson, Hatai, Jackson, Lowrey, and others are also available for comparison.

During the experiment the rats were kept each in a separate cage with wire-net bottom, allowing the feces and urine to drop through, which might otherwise be eaten. Both feces and food were carefully weighed. The rats were individually weighed daily before feeding. Those under experiment were allowed dry food *ad libitum*, together with whole milk carefully regulated in amount so as to hold them nearly constant at the initial body weight. Of course, slight fluctuations in the gross body weight were unavoidable, but they rarely exceeded 2 grams above or below the initial weight, as shown in table 1. The final gross body weight is in all cases nearly the same as the initial weight.

The temperature of the animal room was fairly constant at about 75° Fahrenheit (extreme range 65° to 80°).

Various methods of feeding were used. The eight initial controls were killed shortly after weaning (which occurs at the age of about three weeks), being subsequently fed on Graham bread and whole milk. Two control rats (K5.2, K8.5) were fed with dry 'dog-biscuit' (composition given by Kudo, '21) and water *ad*



libitum, together with 5 to 15 grams of whole milk daily. One control rat (K5.2) was fed with maize (Indian corn) with milk and water. These grew normally up to the ages indicated in table 1.

The test rats (held at nearly constant body weight) were variously fed as follows:

1. Twenty rats (including all the test rats except those specified below) were fed with dry 'dog-biscuit' ad libitum. They consumed an average of about 3.7 grams daily. The amount of milk fed daily decreased from about 3.5 to 4 grams at first to less than 2 grams after three or four weeks. In experiments extending more than a month, however, it was necessary to increase the amount of milk again to 2.5 or 3 grams daily. Feces averaged about 0.8 gram daily throughout the experiment.

2. Five rats (K14.5, K14.9, K10.9 K13.3 and K10.8) were fed with crushed maize. Each rat consumed daily about 3.5 grams. The amount of milk fed decreased from about 3.5 to 4 grams daily at first to about 1 gram after three or four weeks. The feces averaged 0.7 gram daily.

3. Two rats (K14.4 and K14.3) were fed with a mixture of crushed maize and 'dog-biscuit.' They ate about 3.6 grams daily of that mixture and were fed about the same amount of milk as in (2). The feces averaged about 0.8 gram daily.

4. Two rats (K11.3 and K12.1) were fed with milk powder ('Klim' brand). They consumed a daily average of about 3.4 grams of the dry powder. In addition fresh whole milk was fed, decreasing from about 5 grams at first to less than 2 grams after a month.

5. One rat (K14.2) was fed with dried polished rice. It daily ate 3.8 grams of rice throughout the experiment and passed an average of 0.7 gram of feces. At first, milk was added to the diet as follows. In the first two weeks of the test the rat was fed milk in amounts decreasing from 1.8 to 0.7 gram daily; then for about three weeks with 0.5 to 0.1 gram of milk daily. In the final period of four weeks the rat was fed with dry rice only. In that period the body weight was reduced from 33.5 grams to 27.1 grams. This rat was very active and showed no evident symptoms of vitamine deficiency.

TABLE 1  
*Individual data on rats used*

RAT NUMBER AND SEX	FINAL GROSS BODY WEIGHT (AND RANGE)	FINAL NET BODY WEIGHT	BODY LENGTH	AGE OF RAT, DAYS	
				Initial	Final

A. Normal controls					
	grams	grams	cm.		
K12.3 f	22.7	20.5	8.8	25	
K12.2 f	24.0	22.6	9.4	25	
K11.0 f	25.4	23.4	9.6	31	
K 8.1 f	29.3	23.7	9.9	32	
K 5.1 m	27.8	27.2	11.1	26	
K14.0 m	28.1	26.0	9.8	30	
K 7.1 f	36.4	34.4	10.5	35	
K 9.1 m	43.5	41.2	12.0	40	
K 5.3 m	115.7	108.2	16.2	24	56
K 5.2 f	121.5	114.5	16.8	28	77
K 8.5 f	146.5	142.5	18.4	28	112

B. Test rats (held nearly at maintenance)					
K11.4 f	19.9 (19.9-26.2)	18.5	8.8	27	33
K13.2 f	21.9 (21.9-25.0)	18.9	9.0	27	33
K11.3 f	21.9 (21.9-26.8)	20.5	9.7	28	34
K14.1 f	25.4 (25.0-28.8)	22.9	9.3	30	41
K 8.2 m	29.1 (27.7-29.9)	24.5	9.5	32	47
K 8.3 m	29.6 (25.8-31.0)	26.3	9.7	32	50
K14.4 m	23.0 (23.0-30.7)	21.1	9.3	28	49
K14.7 m	23.7 (23.7-32.1)	21.6	8.7	29	49
K14.8 m	25.6 (23.2-30.7)	22.6	10.1	29	51
K14.10 f	26.8 (24.7-31.6)	23.3	10.3	32	53
K14.5 m	26.6 (25.7-30.0)	23.4	9.9	28	50
K14.9 f	26.1 (25.5-31.0)	23.7	10.4	30	50
K14.3 m	24.7 (24.2-33.9)	22.7	9.5	28	54
K10.9 m	25.6 (23.6-30.0)	23.0	10.4	29	58
K14.6 f	28.3 (24.8-31.5)	25.6	9.4	28	62
K10.6 m	23.1 (23.1-28.4)	20.1	9.1	28	65
K10.3 m	26.6 (26.6-31.8)	23.9	10.5	28	65
K13.3 f	24.9 (22.6-29.3)	22.6	8.9	32	71
K12.1 f	25.3 (23.8-30.5)	23.6	9.0	25	67
K 5.4 m	36.2 (31.1-36.8)	33.3	11.3	33	79
K10.4 m	25.4 (24.0-28.7)	23.1	10.1	28	74
K12.5 f	28.3 (26.0-29.2)	24.1	10.3	28	76
K10.1 f	28.7 (28.1-31.9)	26.8	10.7	28	77
K10.2 f	30.1 (26.6-31.8)	25.7	10.5	28	78
K10.8 f	24.6 (23.6-29.6)	22.6	8.9	28	87
K 8.4 f	30.3 (25.2-32.1)	26.8	9.8	32	95
K14.2 f	26.9 (26.9-36.7)	25.0	10.5	28	95
K 9.2 m	43.5 (38.2-49.0)	39.1	11.9	40	104
K10.5 f	26.0 (23.7-26.8)	21.9	9.7	28	104
K12.4 f	28.1 (26.3-32.0)	25.8	10.4	28	117

While no exact chemical determinations were made, it is evident that the amounts of food consumed by the rats in these thirst experiments are more than sufficient for maintenance when water is allowed *ad libitum*. Under these circumstances, Jackson ('15) found that rats of about 24 grams in body weight require for maintenance only about 5 grams daily of Graham bread soaked in whole milk; decreasing to about 3 grams later. Since a greater amount of food was consumed by my rats on the thirst experiments, it is evident that the prevention of growth is due to the lack of water rather than to inanition from inadequate food-intake.

In the inanition experiments with water (Jackson, '15 a) the amount of food required for maintenance of body weight in young rats was found to decrease as the experiment proceeds. Similarly in the present tests (as above shown) a decreasing amount of liquid (milk) is required for maintenance with dry food *ad libitum*, although the amount of dry food taken remains fairly constant.

The difference in the amount of milk required for maintenance of body weight with the different kinds of food probably depends (at least in part) upon the amount of water contained in the food. In all cases, however, there is a decrease in the milk required as the experiment proceeds. Jackson ('15 a) thought that in young rats held at maintenance by underfeeding the amount of living protoplasm is greatly decreased, thereby decreasing the basal rate of metabolism, with a corresponding decrease in the necessary food-intake. Possibly a similar explanation may hold in the case of the present thirst experiments. The metabolism may be altered in some way so as to require less water for maintenance of constant body weight.

Both test rats and controls were killed by chloroform and dissected according to the technique described by Donaldson ('15), with a few modifications. The submaxillary gland and thyroid gland were removed first. The parts and organs upon removal were placed in a closed jar upon glass plates resting on moist filter-paper. After weighing, the organs were dried to constant weight in an oven at about 95°C. in order to determine their water content (not included in this paper).

Percentage losses in the various organs were calculated as follows. The average weight (table 2) for each organ or part in the test rats was compared with the corresponding average for the normal controls of similar body weight. The data in the column 'Difference' express the apparent percentage changes (+ or -) as the result of the experiment. In table 2 the average gross body weight and net body weight (gross body weight minus intestinal contents) are seen to differ but slightly (usually less than 5 per cent) in the various test groups, as compared with the normal controls. These differences have been ignored in calculating the percentage changes for the various parts.

In view of the comparatively small number of observations and the known variability, especially of some of the organs (Jackson, '13), the conclusions reached in the present paper are by no means to be considered as final. It is believed, however, that they are sufficient to give an approximate idea of some of the more obvious and important changes, so as to make possible a comparison of the effects of a relatively dry diet (water deficiency) with other forms of inanition.

#### GENERAL OBSERVATIONS

In general the test rats remained active and apparently healthy, although many died during the course of the experiment, as above stated. The skin becomes somewhat roughened, but the hair is not easily detached (as occurs in adults during thirst). Dryness and desquamation were observed on the plantar surfaces. The claws become much elongated, especially in the later test periods. The fecal material is usually hard, never diarrheal in character. The urine is scanty. Haemorrhage from the conjunctiva or nose was not observed, although it sometimes occurred in the stomach or intestines. In the rats dying during the experiments (not included in the tables) hemorrhage and ulceration of the stomach were often observed. Dryness of the external genital organs, especially in females, was observed, and eczematous conditions often occurred in the longer experiments. Paralysis of the legs or other parts was never found in the young rats (as noted occasionally in adults by Kudo, '21). In some young rats the



TABLE 2  
Average data for controls and test rats, with percentage of change in the various test groups, as compared with normal controls.  
Weights are given in grams, lengths in centimeters

	NORMAL CONTROLS (2m; 4f) AGE 25 TO 30 DAYS. AVERAGE	TEST RATS ON DRY DIET HELD AT CONSTANT BODY WEIGHT FOR PERIODS INDICATED				
		1 to 2 weeks (2m; 4f) Average and percentage difference	3 to 4 weeks (6m; 2f, Average and percentage difference	5 to 6 weeks (3m; 3f) Average and percentage difference	7 to 8 weeks (1m; 4f) Average and percentage difference	9 to 13 weeks (0m; 4f) Average and percentage difference
Gross body weight....	26.2	24.6 (-6.1)	25.3 (-3.4)	27.4 (+4.6)	27.4 (+4.6)	27.8 (+4.6)
Net body weight.....	23.7	22.4 (-5.5)	22.7 (-4.2)	24.9 (+5.1)	24.4 (+3.0)	24.9 (+5.1)
Body length.....	9.8	9.3 (-5.1)	9.8 ( 0)	9.9 (+1.0)	10.1 (+3.1)	10.1 (+3.1)
Tail length.....	7.0	7.4 (+5.7)	8.7 (+24.3)	8.4 (+20.0)	8.8 (+25.7)	9.0 (+28.6)
Integument.....	4.35	3.74 (-14.0)	3.91 (-10.2)	3.48 (-10.0)	3.97 (-8.7)	3.98 (-7.5)
Musculature.....	7.32	6.81 (-7.0)	6.98 (-4.7)	7.38 (+0.8)	6.82 (-6.8)	6.97 (-4.8)
Lig. skeleton.....	3.73	4.26 (+14.2)	4.59 (+23.1)	5.09 (+36.5)	5.22 (+40.0)	5.06 (+35.7)
Cart. skeleton.....	2.79	3.80 (+36.2)	3.42 (+22.6)	4.17 (+49.5)	4.29 (+53.8)	4.42 (+58.4)
Humeri and femurs...	0.3355	0.3856 (+14.9)	0.4026 (+20.0)	0.4540 (+35.3)	0.4152 (+23.7)	0.4439 (+32.3)
Visceral group.....	5.1912	5.3501 (+3.1)	5.4883 (+5.7)	5.6224 (+8.3)	6.1769 (+19.0)	6.2578 (+20.6)
Remainder.....	1.10	0.51 (-53.7)	0.58 (-47.3)	0.64 (-41.9)	0.59 (-46.4)	0.54 (-50.9)
Brain.....	1.2424	1.1894 (-4.3)	1.3088 (+5.4)	1.2583 (+1.3)	1.2636 (+1.7)	1.3493 (+8.6)
Spinal cord.....	0.1565	0.1891 (+20.8)	0.2107 (+34.6)	0.2165 (+38.2)	0.2389 (+52.5)	0.2398 (+53.1)
Nn. ischiadici.....	0.0087	0.0090 (+3.5)	0.0119 (+36.8)	0.0111 (+27.6)	0.0123 (+41.4)	0.0129 (+48.3)
Eyeballs.....	0.1054	0.1152 (+9.3)	0.1456 (+38.8)	0.1448 (+37.5)	0.1719 (+63.8)	0.1797 (+70.8)
Heart.....	0.1696	0.1557 (-8.2)	0.1547 (-8.8)	0.1695 (+0.06)	0.1963 (+15.8)	0.1873 (+10.4)
Spleen.....	0.0838	0.0432 (-48.4)	0.0474 (-43.5)	0.0436 (-48.0)	0.0762 (-9.1)	0.1136 (+36.6)
Lungs.....	0.2369	0.2145 (-9.5)	0.2310 (-2.5)	0.2452 (+3.5)	0.2363 (-0.25)	0.0523 (-37.6)
Parotid glands.....	0.0284	0.0149 (-47.6)	0.0152 (-46.5)	0.0151 (-59.6)	0.0160 (-43.7)	0.2294 (-2.8)
Submaxillary glands..	0.1010	0.0759 (-24.9)	0.0573 (-43.3)	0.0415 (-59.0)	0.0512 (-49.3)	0.0155 (-45.4)
Liver.....	1.1636	1.3244 (+13.8)	1.2313 (+5.8)	1.3045 (+12.1)	1.2363 (+6.3)	0.0598 (-40.8)
Pancreas.....	0.1389	0.1610 (+15.9)	0.1795 (+29.2)	0.1722 (+24.0)	0.1846 (+32.9)	1.5522 (+33.5)
						0.2032 (+46.3)

Stomach-intestines (with contents).....	3.60	3.65 (+1.4)	3.94 (+9.4)	4.00 (+11.1)	4.65 (+29.2)	4.70 (+30.6)
Stomach-intestines (empty).....	1.30	1.38 (+6.2)	1.36 (+4.6)	1.44 (+10.8)	1.68 (+29.3)	1.75 (+34.6)
Kidneys.....	0.2988	0.4034 (+35.0)	0.4451 (+48.4)	0.4797 (+60.5)	0.4940 (+65.3)	0.4435 (+48.4)
Testes.....	0.1427	0.0962 (-32.5)	0.0748 (-47.5)	0.1005 (-29.5)	0.0450 (-68.3)	
Epididymides.....	0.0190	0.0144 (-24.2)	0.0147 (-22.6)	0.0128 (-32.6)	0.0132 (-30.5)	
Ovaries.....	0.0114	0.0077 (-32.5)	0.0061 (-46.5)	0.0041 (-64.0)	0.0043 (-62.3)	0.0038 (-66.7)
Uterus.....	0.0161	0.0138 (-14.3)	0.0174 (+8.1)	0.0136 (-15.5)	0.0204 (+26.7)	0.0217 (+34.8)
Thyroid.....	0.0038	0.0033 (-13.1)	0.0035 (-7.9)	0.0032 (-15.8)	0.0028 (-26.3)	0.0039 (+2.6)
Thymus.....	0.0771	0.0240 (-68.9)	0.0094 (-87.8)	0.0134 (-82.6)	0.0109 (-85.9)	0.0067 (-91.3)
Suprarenals (m).....	0.0077	{ 0.0124 (+61.0)			0.0132 (+71.4)	
Suprarenals (f).....		{ 0.0106 (+37.7)			0.0128 (+66.2)	
Hypophysis (m).....	0.0016	{ 0.0017 (+6.3)			0.0023 (+43.8)	
Hypophysis (f).....		{ 0.0015 (-6.3)			0.0022 (+37.5)	

penis was extruded, possibly from attempts to get the urine. It is to be noted that the specific symptoms generally found associated with various vitamin deficiencies are either slight or entirely absent in survivors of these thirst experiments.

The general condition observed at autopsy are as follows: There is extreme general emaciation, especially in the longer periods. The skin and muscles appear somewhat dry and difficult to separate. The blood is thick. The fat has usually almost disappeared from the subcutaneous and muscular tissues; but in some cases it remains in relatively large amount. The orbital and interscapular fat persists in small amount even in the longer experiments. There is no appreciable change in amount of serous fluid in the pericardial, pleural, and peritoneal cavities. The viscera generally appear normal. The liver is often congested and occasionally yellowish in color (fatty change?). The kidneys are often congested, and their surfaces appear rough in a few cases. Marked congestion of the brain, kidneys, suprarenals, spleen, and lungs was sometimes found. In general, however, the effects of thirst on young rats, as shown by observations and autopsy, appear slighter than in adult rats.

#### LENGTH OF BODY AND TAIL

The body length is measured from the tip of the nose to the anus and the tail length from the anus to the tip of the tail. The measurements were taken immediately after death, the body and tail being extended by very slight tension. Measurements during life are not very accurate, although they might be obtained by the use of anesthetics. Some measurements were made on the living animals, however, which show changes in the lengths of body and tail similar to the changes indicated by the measurements at autopsy.

In the groups of rats held at constant body weight for the five periods (table 2) the percentage changes in average body length are insignificant, usually corresponding to the slight differences in body weight between test rats and controls. It is evident that the body length remains nearly constant. The tail length, however, shows a marked and progressive increase, indicating for the

five periods gains of 5.7, 24.3, 20.0, 25.7, and 28.6 per cent, respectively (table 2).

Thus the rats held at constant body weight by thirst become relatively long-tailed, as found likewise by Jackson ('15 a) in young rats held at maintenance by underfeeding. Some data indicating an opposite result were reported by Hatai ('08).

In adult rats during thirst the body length in the acute series shows an average loss of 11.4 per cent and in the chronic-thirst series a loss of 14.7 per cent. The tail, however, has nearly the same average length in controls and test rats (Kudo, '21).

#### INTEGUMENT

The general changes in the integument were mentioned above under 'General Observations.' In the young rats held at constant body weight in five test series (table 2) there is a slight apparent loss varying from 7.5 to 14 per cent in weight of the integument (which includes the skin and appendages, hair and claws). It would appear that this slight loss occurs early, and is not progressive during the course of the experiment.

In adult rats during thirst the loss of the integument is very nearly proportional to that of the whole body (Kudo, '21).

In inanition (with water) a much greater loss of 36 per cent or more was found in the weight of the integument of young rats by Jackson ('15 a).

#### SKELETON

The bones, together with the cartilages, periosteum, and ligaments, constitute the 'ligamentous skeleton.' The bones and cartilages, after removal of the periosteum and ligaments by immersion for about one hour in 1 per cent aqueous 'Gold Dust' (a commercial soap powder) solution at 90°C., constitute the 'cartilaginous skeleton.' Donaldson and Conrow ('19) have shown that while such a maceration in hot 'Gold Dust' solution causes only a slight loss (less than 5 per cent) in the skeleton of adult rats, in young rats the loss in skeletal weight is considerably greater, amounting to about 15 per cent in the new born. The humerus and femur (of both sides) were therefore cleaned sepa-



rately without maceration, and their weights are recorded separately in table 2, though also included in the weights of the whole skeleton.

As shown by table 2, while the body weight is held constant during the thirst experiments, the ligamentous skeleton continues to increase in weight to a marked degree. The increase is progressive from 14.2 per cent at one to two weeks up to 40 per cent at seven to eight weeks (slightly less at nine to thirteen weeks). For the cartilaginous skeleton the apparent increase in weight is still greater, ranging from 22.6 to 58.4 per cent. It is possible that these large percentages (especially that of 36.2 per cent at one to two weeks) may be erroneous, due to abnormalities or to errors in technique.

The humerus and femur (weighed separately in the moist cartilaginous state, after removal of periosteum and ligaments without immersion in the hot soap solution), as shown in table 2, show percentage increases which are very close to those of the ligamentous skeleton, and these probably form a more accurate index of the actual changes in the weight of the cartilaginous skeleton.

In an underfeeding experiment, Jackson ('15 a) found that the increase in young rats held at constant body weight from three to ten weeks of age forms 28 per cent in the weight of the ligamentous skeleton, and 21.5 per cent in the weight of the cartilaginous skeleton which is somewhat lower than that obtained in the present thirst experiments. This may be because less water is required for the growth of bone, so that its growth is less retarded on a relatively dry diet.

In the inanition experiment on young rats, Jackson ('15 a) noted that the skeletal growth tends to proceed along the lines of normal development, as indicated by decrease in the water content, by formation and union of various epiphyses, etc. Very similar phenomena were noted in the skeleton during the present thirst experiments (epiphyses of vertebrae and humerus, appearance of third molars, etc.).

Data cited by Jackson from other investigators (Waters, Aron, Variot) indicate that a persistent growth of the skeleton during

inanimation likewise occurs in the young of other species (calves, puppies, human infants). Jackson and Stewart have shown that the growth of the skeleton in underfed rats appears less intensive in very young (newborn) rats and in older rats (approaching maturity).

#### MUSCULATURE

As shown in table 2, the various groups of tests rat show apparent slight decreases (4.7 to 7 per cent) in weight, excepting the third group, which is nearly constant. These differences are insignificant, especially when the slight differences in body weight between controls and test rats are taken into account, but there is perhaps a slight tendency to decrease in the later periods.

In young rats held at constant body weight by inanition (simple underfeeding) the musculature, as in thirst, also remains nearly constant, but with a very slight tendency to increase in weight (Jackson, '15 a).

During thirst in adult rats the musculature loses approximately in proportion to the entire body (Kudo, '21).

#### VISCERA AND 'REMAINDER'

The visceral group includes the brain, spinal cord, hypophysis, and eyeballs, as well as thoracic and abdominal viscera. In the animals held at constant body weight (table 2) the visceral group shows a progressive increase in weight of 3.1 to 20.6 per cent in the five test series.

The weight of the visceral group depends essentially upon that of the larger organs. As will be seen later, however the individual viscera differ greatly in their changes in weight during the thirst experiments.

In the maintenance of the body weight by simple inanition, Jackson ('15 a), in young albino rats, and Aron ('11), in young dogs, found that the visceral group undergoes but little change in weight.

The 'remainder' is that part of the body which remains after removing the skin, skeleton, musculature, and visceral group. It includes the adipose and interstitial connective tissue, mesen-

terium, larger peripheral nerves, and blood-vessels, etc. The escaped fluids and the loss by evaporation are not included. As shown in table 2, the 'remainder' in the various tests undergoes a loss in weight increasing from 37.3 per cent in the one to two weeks' test to 50.9 per cent in nine to thirteen weeks. This loss is probably chiefly due to that of the fat, which largely disappears, as mentioned under 'General Observations.'

#### BRAIN

The brain weight of the control rats (table 2) corresponds closely to the data of Donaldson ('15) and Jackson ('15a). In the rats at the various test periods the average brain weight shows slight apparent changes, varying from -4.3 per cent to +8.6 per cent. It will be noted, however, that (with one exception) these apparent changes are in the same direction as the corresponding slight differences between test rats and controls in average body weight. The apparent differences in brain weight are therefore insignificant, considering the small number of observations, and it seems probable that the brain undergoes no appreciable change in weight during these thirst experiments.

Hatai ('04) found a slight apparent loss (average about 4.7 per cent) in the weight of the brain in young albino rats fed with starch, beef fat, and water, the body weight being reduced about 30 per cent (average). In a later experiment, however, Hatai ('08) found that the growth in brain weight was retarded in the same proportion as the whole body weight. In albino rats with retarded growth on a lipoid-free ration, the brain was apparently about 2 per cent subnormal in weight (Hatai, '15).

In underfed young rats (with water), Donaldson ('11) found an apparent slight increase (3.6 per cent) in the brain weight, while Jackson ('15 a) found no significant change. It is evident that the age at which the inanition occurs is important, as Stewart ('18, '19) found a marked increase (125 per cent) in the brain of newborn rats held at constant body weight by underfeeding for sixteen days. Variot and Lassabliere ('09) observed a tendency to persistent growth in brain weight in human infants whose body weight was retarded by malnutrition. The brain

weight of the adult rats subjected to thirst showed no significant change in weight (Kudo, '21). The constancy of the brain weight in adults under various forms of inanition has been repeatedly observed by numerous investigators. McCarrison ('19), however, finds an apparent increase of one-seventh in the brain weight of monkeys on various diets deficient in vitamins.

#### SPINAL CORD

The spinal cord shows a marked and progressive increase (20.8 to 53.1 per cent) in weight in the various test periods (table 2).

Jackson ('15 a) found an increase of 36 per cent in the spinal cord of rats held at constant body weight by underfeeding from three to ten weeks of age, and a smaller increase was obtained by Donaldson ('11). In newborn rats, held at maintenance by underfeeding from sixteen days, Stewart ('19) found an increase of 83 per cent in the weight of the spinal cord. In the thirst experiments on adult rats, the spinal cord shows but little change in weight (Kudo, '21).

#### SCIATIC NERVES

The sciatic nerves (nn. ischiadici) in the test rats showed an increase in weight which is similar to that of the spinal cord, excepting the first period (table 2). This indicates that the continued growth of the spinal cord is correlated with that of the peripheral nerves, as might be expected. In the thirst experiments on adult albino rats, however, the sciatic nerves lost in weight, while the spinal cord did not. No further data on the changes in weight of peripheral nerves during inanition have been found in the literature.

#### EYEBALLS

The eyeballs likewise show a marked and progressive increase in average weight during the various test periods (table 2), varying from 9.3 to 70.8 per cent. The apparent increase of 70.8 per cent in weight of the eyeballs during the thirst experiments is greater than that observed in any other organ or system.



In the adult rats the eyeballs lose nearly 10 per cent during thirst (Kudo, '21). In young rats underfed (with water) from three to ten weeks of age, Jackson ('15 a) found an increase of nearly 50 per cent in the weight of the eyeballs. A still greater increase (146 per cent) was found by Stewart ('19) in similar experiments on newborn rats.

Jackson ('15 a) thought the striking growth capacity of the eyeballs during inanition might depend upon their large water absorption, as the eyeballs are known to have a very high water content. The present thirst experiments, however, indicate that the remarkably persistent growth of the eyeballs continues even when the water supply to the organism is greatly restricted.

Notwithstanding the great increase in the size of the eyeballs in the test rats, they do not protrude abnormally. More space is doubtless provided for them, partly by actual growth of the skeletal orbit and partly by atrophy of the orbital fat.

#### HEART AND AORTA

The heart (table 2) in the test rats shows an apparent slight decrease in weight in the earlier periods with a small increase in the later periods of thirst. It will be noted, however, that these changes do not greatly exceed the differences between test rats and controls in body weight, which are in the same direction. The apparent changes in heart weight are therefore of doubtful significance.

In rats underfed from three weeks of age, Jackson ('15 a) likewise found no significant change of weight, although in underfed newborn rats Stewart ('19) noted an increase of 26 per cent.

During thirst in adult rats the heart likewise maintains its relative weight, losing in absolute weight nearly in proportion to the entire body (Kudo, '21).

*Aorta.* The aorta was cut proximally at the heart and distally at the origin of the iliac branches. All branches of the aorta were clipped close to the vessel. All blood content was removed. The following observations are too few (especially on controls) to warrant conclusions, but would seem to indicate an increase in the weight of the aorta during the thirst experiments.

LENGTH OF TEST	NUMBER OF RATS	AVERAGE NET BODY WEIGHT	AVERAGE WEIGHT OF AORTA
		<i>grams</i>	<i>gram</i>
(Control)	1	26.0	0.0187
1-2 weeks	2	20.9	0.0159
3-4 weeks	7	22.6	0.0212
5-6 weeks	3	23.9	0.0246
7-8 weeks	4	24.8	0.0232
9-13 weeks	3	24.2	0.0234

## SPLEEN

The spleen (table 2) shows a marked loss in average weight (36.6 to 48.4 per cent) in all the test groups but one, in which the loss appears much smaller (9.1 per cent). The very small apparent loss in this test group is due to an abnormally large spleen which weighed 0.2 gram, which brought up the average for the group. If this abnormal spleen were excluded, the loss in this group would appear as 46.5 per cent. Similarly in the last group, the inclusion of an abnormally large spleen gives an apparent average increase of 36.6 per cent; omitting this spleen, there is an average loss of 37.6 per cent. Although the spleen is normally one of the most variable organs in the body (Jackson, '13), requiring caution in drawing conclusions, it is evident that it usually undergoes a great loss in weight in young rats held at constant body weight by thirst.

In adult albino rats during thirst, both acute and chronic, the spleen similarly loses relatively much more in weight than does the body as a whole (Kudo, '21). Jackson ('15 a) found that in young rats held at maintenance by underfeeding beginning at the age of three weeks there is a marked tendency to decrease in weight of the spleen, while at later (and longer) periods the spleen appears to undergo no material change in weight. In the underfed newborn rats, Stewart ('19) found an increase of 33 per cent in the spleen.

## LUNGS

The lung infections frequently found in older rats rarely occur before the age of ten weeks (Jackson, '15 a) and did not occur in the rats shown in tables 1 and 2. The average weight of the lungs in the test groups shows slight changes, varying from  $-9.5$  to  $+3.5$  per cent. These changes are usually in the same direction as the differences in average body weight, however, and are too small to be significant. It is therefore evident that there is but slight if any change in the weight of the lungs during the present thirst experiments.

During thirst in adult rats the percentage loss in the weight of the lungs appears slightly greater than that of the whole body (Kudo, '21). In young rats held at constant body weight by underfeeding, Jackson ('15 a) found a slight decrease in the early periods, but not in the later. There is no appreciable increase in the lungs of the underfed newborn rats (Stewart, '19).

## SALIVARY GLANDS

*Parotid glands.* The parotid glands (table 2) show a marked decrease in average weight, varying from 43.7 to 59.6 per cent in the various test groups. This loss apparently occurs early and remains fairly uniform throughout the various test periods. In adult albino rats during thirst, both in acute and chronic, the percentage loss in weight of the parotid glands is likewise much larger than that of the entire body (Kudo, '21).

*Submaxillary glands.* A striking decrease in weight similar to that of the parotid glands is apparent in the submaxillary glands (table 2) in all except the first test group, where the loss (24.9 per cent) is somewhat smaller. In adult albino rats during thirst, both acute and chronic, the submaxillary glands likewise decrease in weight relatively much more than does the body as a whole (Kudo, '21).

## LIVER

The liver shows somewhat irregular increases in average weight (table 2), varying from 5.8 to 33.5 per cent in the different test periods. While the liver thus shows a definite tendency to

increase, caution must be observed in drawing conclusions, on account of the great variability in the weight of the normal liver (Jackson, '13).

In the adult rats during thirst the liver loses weight in about the same proportion as the whole body (Kudo, '21). In young rats underfed from three weeks of age the liver is variable, showing a definite increase in weight in the earlier periods, but a decrease later (Jackson, '15 a). Stewart ('19) found a marked loss in the liver weight of underfed newborn rats.

#### PANCREAS

The pancreas (table 2) in the various test periods shows a progressive increase in average weight (15.9 to 46.2 per cent). It may be noted that the average weight of the pancreas in my controls (0.1389 gram) is considerably below the corresponding weight found by Hatai ('18) (0.206 gram); but, as Hatai remarks, it is difficult to dissect out the gland with uniform accuracy.

In adult rats a decrease in weight of the pancreas during thirst is relatively much greater than that of the whole body. Thus the loss in the weight of the pancreas resembles that of the salivary glands.

#### STOMACH AND INTESTINES

The stomach and intestines were separated from mesentery and pancreas. The digestive tube with contents (table 2) shows a progressive increase in average weight, varying from 1.4 to 30.6 per cent. The increase is not significant until after six weeks. The data for the empty stomach and intestines show a very similar progressive increase in average weight, varying from 4.6 to 34.6 per cent. Thus both the alimentary canal and the intestinal contents appear to increase in weight, especially in the thirst experiments extending beyond six weeks in length. The intestinal contents in the test rats are watery or mucous in character in the small intestine, becoming usually very hard in the fecal material of the large intestine.

In rats underfed from three weeks of age Jackson ('15 a) likewise found an increase in the weight of the intestinal canal (plus



mesentery and pancreas), which continued up to the age of six weeks, but appeared to decrease later. In adult abline rats during thirst there is a marked decrease (relatively slightly less than that of the entire body) in the weight of the stomach and intestines (Kudo, '21).

#### KIDNEYS

The kidneys (table 2) in the test groups show a marked apparent increase in average weight, progressing from 35 to 65.3 per cent in the first four groups, but decreasing to a gain of only 48.4 per cent in the longest test (nine to thirteen weeks). It is possible, however, that the large apparent increase may be due in part to an abnormally low kidney weight in my controls, which is somewhat below the normals of Jackson ('15 a) and Donaldson ('15). In young rats underfed beginning at three weeks of age Jackson ('15 a) found a slight increase in the weight of the kidney, but little or no change at later periods. Stewart ('19) found a great increase (90 per cent) in the kidney weight of underfed newborn rats.

In adult rats during acute and chronic thirst the kidneys lose in weight relatively slightly less than the body as a whole (Kudo, '20).

#### TESTES

The average net body weights of the males in the various groups of table 2 are as follows: controls (2), 26.6 grams; 1-2 weeks (2), 26.7 grams; 3-4 weeks (6), 22.4 grams; 5-6 weeks (3), 25.8 grams; 7-8 weeks (1), 23.1. It is thus evident that the average body weights of the various test groups differ somewhat from the controls, which partly accounts for the greater apparent losses in the groups at three to four weeks and seven to eight weeks. Making allowance for this difference in body weight, the tests would show an average decrease of something over 30 per cent.

On the other hand, Jackson ('15 a) found an apparent increase of 34 per cent in the weight of the testis in rats underfed beginning at three weeks of age, and Stewart ('19) obtained an enormous increase (average 374 per cent) in the testes of newborn rats held at maintenance by underfeeding for about sixteen days.

In adult rats the testes during acute thirst lose about 15 per cent in their weight and during chronic thirst they lose 52.7 per cent, while the loss of the whole body is respectively 36.1 per cent and 52.4 per cent (Kudo, '21). Hatai ('15) found that the testes of young albino rats show an actual loss in weight (23 per cent) as a result of six months on the lipid free-diet.

#### EPIDIDYMIDES

The apparent losses in the average weight of the epididymis (table 2) are, of course, subject to the same corrections as those of the testis, on account of differences in body weight between test rats and controls. The actual losses are therefore somewhat lower than those indicated in the table and below those of the testis.

In adult rats during thirst the epididymides lose weight roughly in proportion to the body weight (Kudo, '21).

#### OVARIES

The average body weight of the female rats in the control group (table 2) is slightly lower than that in all the test groups, excepting the first. But the maximum difference in body weight is only about 10 per cent, so the relatively large apparent losses in the average weight of the ovaries, increasing progressively from 32.5 to 66.7 per cent, would not be materially affected by the required corrections.

This loss in the weight of the ovaries during thirst is materially greater than that (27 per cent) found by Jackson ('15 a) in rats underfed beginning at three weeks of age. Stewart ('19) found practically no change in the weight of the ovaries in underfed newborn rats. Hatai ('15) found in young albino rats fed with lipid-free diet a loss of 17.4 per cent in the weight of the ovaries.

#### UTERUS

The uterus (including tubes) is subject to considerable variation in weight, as appears in the various test groups in table 2. While the tendency during the earlier test periods is somewhat fluctuating and doubtful, there appears to be a definite increase in

average weight (26.7 to 34.8 per cent) in the two longest tests. Its change in weight therefore appears quite different from that of the ovaries.

#### THYROID GLAND

The thyroid gland shows an apparent loss in average weight in all but the last test group (table 2). In most cases the difference is too small to be significant, however, especially when the individual variation in the weight of the thyroid gland and also the great difficulty in dissecting it out in a uniform manner are considered. Jackson ('15 a), however, found an apparent loss of about 24 per cent in the thyroid of rats underfed from three weeks of age.

In adult albino rats during thirst, both acute and chronic, the thyroid glands lose markedly in weight (Kudo, '21).

#### THYMUS

The loss of 68.9 to 91.3 per cent in the average weight of the thymus in the various test groups (table 2) is greater than that in any other organ. According to Hatai ('14), the thymus should reach its maximum absolute weight (0.29 gram) at about eighty-five days of age, after which it normally undergoes a slow age involution. As is well known, the thymus is especially liable to a rapid involution under various unfavorable circumstances ('accidental involution' of Hammar). This was found by Jackson ('15 a) in rats underfed at three weeks of age and later, and also by Stewart ('19) in newborn rats. In adult rats during thirst the involution of thymus is likewise very marked, with loss of about 90 per cent in weight (Kudo, '21).

#### SUPRARENAL GLANDS

In the suprarenal glands of the rat there is normally a sexual difference in weight, observable from the age of about six weeks (Jackson, '13, Hatai, '13). Before this age the sexes may safely be grouped together, as in my controls. In the test groups the sexes are separated, although on account of the small numbers and the irregularity of the data they are combined into only two

age groups, one to four weeks and five to thirteen weeks. All four groups show a marked apparent increase in weight, which is about 60 per cent in all except the younger group of females (37.7 per cent). This difference is not on account of the appearance of the normal sex difference, however, for normally the suprarenals become larger in the female. As a matter of fact, the tendency to sexual difference in weight does not appear in these thirst experiments, (contrary to the observations of Jackson) ('15 a) in underfeeding).

In the rats underfed beginning at three weeks of age Jackson ('15 a) found an apparent increase of 12 to 39 per cent in the weight of the suprarenals. A much smaller increase (5 per cent) was found by Stewart (19) in underfed newborn rats.

In adult rats during thirst there is a relatively small loss in weight of the suprarenal glands (Kudo, '21).

#### HYPOPHYSIS

The hypophysis in rats above 50 grams in body weight must be considered separately in the sexes, since it then normally becomes relatively heavier in the female. This does not affect my controls (grouped together in table 2), and it is evident from table 2 that there likewise appears no sexual differentiation in weight in the test rats during the thirst experiments (in agreement with the inanition experiments of Jackson, '15 a). There is evidently no significant change in the average weight of the hypophysis in the one to four weeks' group, but a marked increase (37.5 to 43.8 per cent) in the five to thirteen weeks' group.

Jackson ('15 a) found a smaller increase (18 to 19 per cent) in the rats underfed from three weeks of age, while Stewart ('19) found a slightly larger increase in the underfed newborn rats.

In adult rats during thirst the hypophysis changes but little in absolute weight (Kudo, '21).

#### DISCUSSION

The changes in the average weights of the various organs and parts in rats held at constant body weight for the various periods are summarized in table 2. While no great emphasis can be laid



upon the exact accuracy of the figures shown in table 2, it is evident that, with respect to the changes in weight during the thirst experiments, the organs may be divided into three groups. In the first group, which includes hypophysis, eyeballs, kidneys, suprarenals, spinal cord, skeleton, sciatic nerves, pancreas, alimentary canal, liver, humerus and femur, visceral group and uterus, there is a well-marked increase in weight during the maintenance of constant body weight by thirst in young rats. As shown in table 2, the rate of increase in the weight of the organs, in general, is progressively greater in the longer periods.

In the second group, which includes heart, brain, musculature, and lungs, the organs remain nearly constant in weight.

In the third group, including the thymus, ovaries, parotid and submaxillary glands, 'remainder,' spleen, testes, epididymides, thyroid, and integument, there is a marked loss in weight. This loss appears in most of the organs already in the earlier test periods and in some cases (thymus, thyroid) appears more or less progressive in character.

If we compare the changes in weight, as a measure of their relative resistance to thirst, in young growing rats with those observed in adult rats during acute and chronic thirst (Kudo, '21), it is found that in general there is in many cases a considerable degree of correspondence. The hypophysis, eyeballs, skeleton, and spinal cord increase in weight in the young rats and also show marked resistance (slight loss in weight) in adults during acute and chronic thirst. The brain in all cases remains nearly constant in weight.

The heart weight is nearly constant in the young rats and loses nearly in proportion to the body during adult thirst. The thymus, 'remainder,' salivary glands, spleen, testes, and epididymides lose weight markedly during thirst in both young and adult rats.

In many other cases, however, the changes in organ weight in the young differ materially in tendency from those in adult. Thus the pancreas and liver have a marked growth tendency in the young test rats, but lose heavily during adult thirst. Lesser degrees of difference are observed in many other organs.

Comparing my results in young rats on thirst tests with those of Jackson ('15 a) in young rats of similar age held at constant body weight by underfeeding (with water allowed), there is found a remarkable similarity between them. Thus, in both cases there is a marked growth in the brain, spinal cord, eyeballs, hypophysis, skeleton, suprarenals, and alimentary canal. In both series the brain, musculature, and heart remained nearly constant, and the thymus, ovaries, spleen, thyroid, and integument decreased greatly in weight. There are some differences between the results of the thirst and the inanition tests, however. The kidneys appear to gain markedly during thirst, but remain constant during inanition. The testes lose heavily in weight during thirst, but gain markedly during inanition. Other forms of partial inanition give results differing more or less from the foregoing, as shown by the experiments of Hatai, Osborne and Mendel, McCarrison, and others on diets defective in various respects, including vitamine deficiencies (cf. Jackson and Stewart, '19, and Kudo, '21).

It has also been shown by Jackson and Stewart that the changes in organ weight during inanition differ greatly according to the age of the animals. Thus, the changes during underfeeding in the newborn are very different from those in adolescent rats and these in turn differ from those in older animals. The age factor is doubtless equally important in the effects of thirst upon the weight of the various organs.

#### SUMMARY

The principal results of the present investigation may be briefly summarized as follows.

Albino rats about one month old may be held at constant body weight for several weeks by a restricted amount of liquid (milk) in a diet otherwise adequate for growth. The rats show a progressive tolerance of thirst, so that less liquid milk is daily required for maintenance as the experiment proceeds.

The tail becomes elongated while the body length remains constant.

There is in general a marked increase in the weight of the skeleton and a slight increase in the visceral group.

The musculature remains nearly constant in weight. There is a slight decrease in the integument and a marked loss in the 'reminder.' Of the individual viscera:

1. The hypophysis, eyeballs, kidneys, suprarenals, spinal cord, skeleton, sciatic nerves, pancreas, stomach-intestines, liver, and uterus show a definite increase in weight.

2. The heart, brain, and lungs remain nearly constant in weight.

3. The thymus, ovaries, parotid and submaxillary glands, spleen, testes, epididymides, and thyroid suffer more or less well-marked decrease in weight.

The growth tendencies of the various organs in the young rats during the thirst experiments correspond in general to those found in rats of similar age during underfeeding, although certain exceptions occur (testes and kidneys).

Likewise the results of the thirst tests (also those of the inanition experiments) show a general resemblance to those of similar character in adults. There are certain differences according to age, however, as well as according to the type of inanition employed.

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Un estudio experimental de los glochidia y los factores que  
provocan su enquistamiento.

La fijación de los glochidia sobre las branquias de los peces depende de una estimulación táctil, mediada por las células pestañosas del manto. La activación química, aun cuando es efectiva en extremo, no existe en el estado natural. La proliferación del tejido de las branquias para cubrir el glochidium y producir un quiste es en principio un proceso reparador que restaura la continuidad del epitelio. La formación del quiste no se inicia o está influida por influencia vital alguna del glochidium, porque puede imitarse aplicando a un filamento branquial pequeñísimas pinzas de metal. No obstante, algún factor regulador normal deja de manifestarse en los experimentos llevados a cabo sobre filamentos separados del animal; el crecimiento excesivo, por consiguiente, produce quistes grandes, malformados, comparables al exceso de proliferación en estos casos después de simple incisión.

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# AN EXPERIMENTAL STUDY ON GLOCHIDIA AND THE FACTORS UNDERLYING ENCYSTMENT<sup>1</sup>

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THREE PLATES (FIFTEEN FIGURES)

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## I. INTRODUCTORY

The young of fresh-water mussels pass the early stages of development within the marsupial gill pouches of the female. Here they progress to a simple, bivalved, larval form, the glochidium, once believed to be a distinct animal parasite and hence for a time termed *Glochidium parasiticum*. In reality, the glochidia are eventually discharged from the gills and settle to the bottom. Further development is conditional upon chance attachment to the gills or fins of appropriate fishes in whose tissues they become encysted, and, as virtual ectoparasites, begin their metamorphosis. After such a period of parasitism, the

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'juvenile' mussel emerges from its cyst and enters upon an independent existence.

This investigation was undertaken to determine, first, the nature and scope of the responsiveness of glochidia to a variety of activating agents. Such a general survey of the sensory-motor range preludes the more particular points of interest, such as the nature of the stimuli leading to natural attachment on the fish host and the induction of cyst formation.

The experimentation was done at the United States Biological Station, Fairport, Iowa, while holding a temporary appointment from the Bureau of Fisheries. Acknowledgment is due the Director, Mr. A. F. Shira, for the facilities of the station, and to the Director, Superintendent H. L. Canfield, Dr. A. D. Howard, and Dr. F. H. Reuling for material. It proved expedient to concentrate attention at first chiefly upon one form, *Lampsilis luteola*, the Lake Pepin mucket; its glochidium is of large size, easily obtainable over long periods, and belongs to the commoner, but less known, hookless group. Later, comparative studies were possible upon other species representing all types.

## II. THE GLOCHIDIUM

*A. Anatomy.* There are two chief types of glochidium, distinguished by the presence or absence of marginal hooks on the valves. It is customary, therefore, to speak of the hooked and hookless glochidia. A third group, either lacking hooks or having peculiar ones, is designated from its appearance when closed as the axe-head type.

*1. Hooked glochidia.* This type is characterized by bluntly triangular and broadly hinged valves, each of which bears a single, stout hook at its apex (figs. 1 and 2). The hooks possess spines on their outer surfaces; when the valves are approximated the hooks are folded inward against the mantle, thus bringing the spines in apposition. It is usually stated that special 'myocytes' retract the hooks, but mutual contract, resultant from the closure of the valves, is by itself quite adequate for this. If the strong glochidial valves have closed on such a soft part as a fin or a gill, it is evident that such an arrangement of hooks and spines

will serve to bring about a firm, locking attachment to the fish host.

This hooked type, although found in but few genera (*Anodonta*, *Strophitus*, *Symphynota*, *Arcidens*, and a few others), is familiar, since it is the one usually figured and described in texts; the commoner hookless type for some reason has been largely ignored. The remaining general points of structure are shared equally by all glochidia and will be considered in the detailed description of the hookless group which follows.

2. *Hookless glochidia*. This far more abundant type of glochidium lacks hooks and has rather delicate valves of a blunt spoon shape (figs. 3 and 4). The actual form and contour of the shells vary widely among the members of currently accepted genera. In size this group ranges from the minute glochidium of *Margaritana*, which measures 0.050 mm. or less, to that of *Quadrula granifera*, 0.290 by 0.355 mm.

The shell, although brittle and weaker than those of the hooked glochidia, is quite firm, and can be subjected to reasonably rough treatment without injury. This is due chiefly to its limy constituents and not to the overlying chitinous cuticula. A thicker ridge of lime is present as a border about the free edge; this shows in external surface view as a double-contoured margin (fig. 3). Along the ventral border of the valves, opposite the ligamentous hinge, is an incurved cuticular flange of considerable transparency (fig. 4). Viewed in profile from the side, it may appear deceptively like a hook. The sharp flange edge serves to bite into the tissues of the host when the glochidium becomes attached.

Of the internal organs, the most conspicuous is the adductor muscle. This, in the opened larva, stretches between the valves as a broad, rounded band (fig. 4). In the closed glochidium it appears in prominent circular outline, placed nearer the future anterior end (fig. 3). The component fibers have the typically elongated nuclei of smooth muscle, but often branch near their insertions on the valves.

Lining the valves is the larval mantle, formed of large, flat cells, and reputed to be active in digesting the enclosed tissue of the host during the early stages of encystment (fig. 4). Cer-



tain of the mantle cells exhibit conical elevations, which project far above their expanded bases. Their apices bear several fine, non-motile bristles. These cells have been designated as 'sensory,' on the basis of suggestive structure and selective stainability with methylene blue (Lillie, '95). Actual continuity or any definite coordinating intermediary between these cells and the muscle fibers, although generally assumed, has never been demonstrated. There are four pairs of such hair cells, symmetrically apposed in the two valves and grouped as outer and inner pairs (figs. 3 and 4). The two outer setae are located about one-third distant from the ventral shell border; they are prominently elevated and can easily be observed in properly illuminated, living glochidia. The two inner pairs are shorter, and are inconspicuous except in stained preparations; the anterior pair is placed much nearer the hinge line than the posterior set.

In a few mussels (*Anodonta*, *Unio*, *Quadrula plicata*, *Quadrula heros*, *Strophitus*) there is an interesting structure, the larval thread, formed by an elongated thread gland. Once believed to be a byssus, and still so designated in many texts, it is in fact an organ peculiar to certain glochidia, and is in no way homologous to the later byssus of the juvenile mussel, formed at metamorphosis. Lillie ('95) has interpreted the thread as a filamentous excretory mass. Shierholz ('88) and others have considered it of use in tangling the glochidia into masses by which many glochidia are drawn into contact with a fish after a single one attaches. That this may be an imperfect explanation is suggested by the observation of Lefevre and Curtis ('12) that the threads of *Anodonta* dissolve within a day or two after the glochidia are free. It is also reported by the same authors that the glochidial thread of *Unio complanatus* is extruded immediately after the larvae are removed from the marsupium, and, according to Harms ('07), in *Margaritana margaritifera* it is lost at a premature stage while the glochidium is still within the egg capsule. It would seem to be of no especial importance mechanically in aiding attachment, but may well be indirectly useful in another way (p. 468).

Just posterior to the adductor muscle, on either side of the median plane, is a symmetrical grouping of cells from which the gut, foot, nephridia, and other organs will develop during metamorphosis (fig. 3).

3. *Axe-head glochidia*. There are four species of mussels, historically placed in the genus *Lampsilis* on the basis of superficial adult shell characters, which possess peculiarly shaped glochidia. These constitute the axe-head type—so-called from their suggestive resemblance in surface view (figs. 5 and 6). The four species producing it have been known as *Lampsilis alata*, *capax*, *laevis*, and *purpurata*, but Simpson ('00) and particularly Sterki ('95, '03), Ortmann ('11), and Utterbach ('16) have elevated them, on the basis of larval characteristics and adult internal anatomy, to a distinct genus, *Proptera*. Here they doubtless belong; at any rate the glochidia deserve a separate grouping. Except for *P. laevis* (Coker and Surber, '11) larvae of the axe-head type possess hooks, which, however, are said by Lefevre and Curtis ('12) not to be homologous with those of *Anodonta* and its allies, but are to be regarded as more nearly related to the hookless forms.

B. *Natural history*. When ripe, the glochidia are free from the egg membranes, and, in most species, are not united with any firmness by the mucus or jelly which binds them into compact 'conglutinates' at earlier stages of development. Indeed, the accepted criterion of 'ripeness' and suitability for artificial infection purposes is based on the degree of freedom of the individual larvae. On the contrary, the glochidia of *Strophitus* are expelled embedded in gelatinous cords and those of *Obliquaria* in cylindrical masses.

According to Lefevre and Curtis ('10, '12), the ripe glochidia, usually held loosely in slimy strings by a mucus secreted from the marsupial epithelium, are discharged through the exhalant siphon at irregular intervals. They sink to the bottom and come to rest with their widely gaping valves open upward. The mucus, when present, is dissolved in a short time, leaving the larvae as entirely separate units.

On reflection, one is struck with a rather uniform result which has been diversely gained in these larval mussels whose further development is dependent on an ectoparasitic sojourn upon an appropriate fish host.

The glochidia of *Unio* and *Anodonta*, supplied with larval threads, are extruded entangled in masses. The hooked but threadless *Symphynota* larvae are spawned within a ropy mucus. *Strophitus* and *Obliquaria* discharge their glochidia in tenacious, gelatinous cords. Most of the remaining glochidial forms are said to be more or less adherent in mucus-bound masses. In these cases, practically without exception, there is a liberation of the larvae to an independent condition soon after being spawned. Thus, the threads of *Unio* dissolve immediately, those of *Anodonta* within a day or two; many of the *Strophitus* glochidia are extruded from the cords within a few minutes after their discharge; the slime of other forms is soon dissolved. Does it not seem reasonable that these relations are each helpful in keeping the larvae together until they have become established on the bottom? Soon becoming free, they would be advantageously aggregated to become whirled up by the respiratory or other currents produced by fishes, attracted, for example, by animal associations about a mussel bed. In this way the chances for a mass infection are better than if the individual glochidia were widely dispersed by currents or other causes. Such an explanation obviates the necessity of assuming, as Howard ('14) has done, that heavy infections presuppose the presence of fish at the time of glochidial extrusion. Although Latter ('91) interpreted his experiments to indicate that the presence of fish does not induce the emission of glochidia, Howard believes that "they support the probability that the approach of fish is the normal stimulus in eliciting the emission of glochidia."<sup>2</sup> On the con-

<sup>2</sup> A curious relation exists between certain mussels and the 'Bitterling,' *Rhodeus amarus*, a cyprinoid fish of central Europe. According to the original account of Olt ('93), at the breeding season the genital papilla of the female *Rhodeus* elongates to a tubular ovipositor about the same length as the fish itself (60 to 80 mm.). The ripened eggs are few, but about 2.5 mm. in size; the ovipositor is introduced between the gaping valves into the mantle cavity of a *unio*



trary, that glochidia may be spawned in the absence of fish is abundantly proved by laboratory observations on mussels held for experimental propagative purposes. Moreover, there is no reason to doubt that expelled glochidia retain for some time their ability to gain attachment, and hence immediate infection is not imperative. Lefevre and Curtis ('12) mention having kept free *Strophitus* glochidia alive for two or three weeks. It, however, does not follow that the larvae are in a favorable physiological condition throughout a correspondingly long period; experience in mussel propagation teaches that good artificial infections are most easily secured within twelve or more hours after removal of the glochidia from the marsupium, yet there is evidence that the fastest infections give inferior yields of young mussels (Dr. H. R. Reuling, private communication).

Exactly what the stimulus may be that induces a mussel to spawn is undetermined. It is interesting to note that such a form as *Lampsilis ligamentina* holds glochidia as long as eight months before liberating them. During this period the larvae are perfectly satisfactory if removed by operation and used for infection purposes. What impels the gravid female eventually to rid itself of larvae awaits explanation.

or anodonta and eggs are deposited between the branchial lamellae; Cuenot ('98) even has a figure showing the ovipositor inserted in the exhalent siphon of the mollusc, "pendant dans les orifices siphonaux!" The male *Rhodeus*, after interesting maneuvers about the mussel, emits seminal fluid near the siphon; the spermatozoa are drawn in through the inhalent siphon, are carried by ciliary currents to the eggs and fertilize them. Development proceeds among the gills for a period of a month, when the fry, now 10 to 11 mm. long, leave their host. Olt states that the breeding seasons of the mussels and fish coincide, and this he believes affords an opportunity for the expelled glochidia to encyst on the fish, brought close by their peculiar breeding habits. Such reciprocal behavior has been stated as a fact by Bridge ('05) and Mitchell ('11). There is, however, nothing in Olt's account to indicate that this possibility was more than an ingenious speculation on his part; Bridge and Mitchell offer no evidence to support their statement, which is doubtless an inference, following Olt. It is conceivable that investigation might prove this relation correct, yet with our recent knowledge of the restriction of parasitism to definite fish hosts it is not as obvious an assumption as in Olt's time; nevertheless, there is clearly no reason for believing that the immediate presence of these fish in any way induces or influences the actual spawning of glochidia.



The statement has often been made that glochidia swim by clapping together the shells, after the manner of *Pecten*. This is, however, wholly erroneous as regards American species.<sup>3</sup> From the time of discharge they lie on the bottom, with gaping valves directed upward, incapable of locomotion, although subject to passive dispersal by water currents.<sup>4</sup> It is true that glochidia, more particularly those of the hooked type, show spontaneous contractions which may continue irregularly for long periods;<sup>5</sup> but these pulsating movements at most cause merely a toppling over of the larva in case the closure is complete.

### III. EXPERIMENTAL

#### A. *Tactile excitation*

The only significant reference to the sensitivity of glochidia to mechanical stimulation is in the report of Lefevre and Curtis ('10, '12). These workers found that the hooked *Symphynota* glochidia respond very readily to the touch of a needle point or paper edge; they continue to clasp such objects until death supervenes. This tactile response was believed to be the chief factor responsible for the attachment of hooked glochidia to the fins or other soft external parts of fishes. As might be expected, those ventral fins which brush the bottom tend to become most heavily infected. Hookless glochidia, however, were said to differ markedly from the hooked type, inasmuch as they "respond

<sup>3</sup> Dr. R. E. Coker writes that he is reliably informed that glochidia of Japanese mussels do swim *Pecten*-fashion; such activity would offer many interesting points for study.

<sup>4</sup> Thus, a variety of glochidia may appear regularly in surface tows of river water (H. W. Clark, unpublished observations), or in water samples from greater depths (Kofoed, '08). This condition possibly explains the natural propagation of such a mussel as the 'nigger head,' *Quadrula ebena*. Its glochidia have a restricted parasitism on the blue herring, *Pomobolus chrysochloris*, which is not a bottom feeder, but predaceous; since, however, both mussel and fish are characteristic of swiftly flowing water, the chances of infection are still good.

<sup>5</sup> In several species of the *Lampsilis* group this was most common directly after removal from the maruspium. Hooked forms may continue spontaneous contractions, at irregular intervals, for days. In *Anodonta corpulenta* the amplitude is great; the opened valves of the larva lie in the same plane, and explosive, winking contraction nearly closes them.

not at all or only sluggishly to tactile stimuli." Hence, in way of summary, these authors conclude that "the stimulus which causes the contraction of the muscle and results in the attachment to the host is, in the case of hookless glochidia, usually a chemical one, but in that of the hooked forms it is mechanical."

1. *Hookless glochidia*. In order to test general tactile sensitivity, and particularly to aid in localizing the sensitive regions, a human eyelash, attached to a holder, was used. Its fine, tapering tip is relatively small, even in comparison to the tiny glochidia, and with it the surface of a larva could be precisely explored. An ordinary hair of the head, with a blunt, cut end, is of too large caliber to be appropriate for this purpose. As will appear later, however, a small stimulating point is not as effective, on the whole, as a larger, blunter surface.

a. *Lampsilis luteola*. The external surface of the valves is entirely insensitive. Even shells which are forced shut and held in a closed position for a time recover fully when the pressure is released.

Touching the mantle appropriately, on the contrary, leads to a very prompt and vigorous closing response. A narrow border zone close to the shell rim is unresponsive (fig. 4). Of the remaining area, it is perfectly obvious that the half adjacent to the hinge is far more sensitive than the rest. But the region of greatest sensitivity lies on the ventral side of the adductor muscle; an open active glochidium, touched properly here, will usually close at the first application. In other, less responsive regions, several attempts may be necessary before the contraction occurs. Closure is in most cases a sudden, vigorous, and uniform snap; with some less active individuals the response is more deliberate. Unless the tactile stimulus is applied as a staccato jab, the hair is caught by active glochidia. Such a prompt and even explosive response is essential to insure attachment under the conditions encountered in nature; contact of a glochidium, for example, with the gills of a fish, is but momentary, due to the swiftness of the respiratory current.

When glochidia, tactilily stimulated, close on a hair, they retain a firm clasp upon it. As a rule, this probably continues until the

death of the larva. Even glochidia, closed by a quick jab of the hair, without the latter's having become caught, still remained shut at the end of fifteen minutes' observation.

The force constituting an effective application with a hair is variable, depending on the region of the glochidium stimulated and the physiological state of the individual. At times, a very delicate touch in the more sensitive region calls forth a prompt response; other lots may be practically unresponsive to the gentlest touches and require a firmer application. With a fine hair the latter condition is probably more common, although it is, of course, possible that the nearer glochidia approach maturity and the time of natural spawning, the lower the threshold to this sort of stimulation becomes. It was observed in some cases, however, that samples of glochidia, which at one time responded fairly sluggishly and closed slowly, were markedly active a few hours later.

It follows from the foregoing statements that these experiments are not in accord with the dictum of Lefevre and Curtis that hookless glochidia "respond either not at all or only sluggishly to tactile stimuli . . . ." The divergence of our conclusions will become more apparent in the pages which follow.

Having thus established the tactile responsiveness of *Lampisilis luteola* as a type of hookless glochidia, the question of the relation of the hair cells to tactile reception next presents itself. The morphological appearance of these columnar elements suggests the strong probability of a sensory activity of some sort, although actual nervous connections with the muscle fibers have never been demonstrated (p. 466); moreover, their position is such that any relatively large object inserted between the gaping valves will impinge upon them (fig. 4).

By properly regulating the illumination the outer set of cells can be seen with ease under high binocular magnification. Especially is this true when the valves are viewed rather obliquely. The inner sets, on the contrary, cannot be identified with sufficient surety to be of use in experimentation. From stained, permanent preparations it is, however, easy to learn their constant asymmetrical location with respect to the adduc-



tor muscle, and these areas can then be found with moderate precision.

Whether or not a closing response follows only when the hair cells are stimulated is not simple to determine. It is certain that one can learn to evade those areas occupied by such sensory cells, and, in most cases, not elude a response. Moreover, if the inner mantle surface is explored by a series of equal staccato prods, it is a common experience to elicit no response for several attempts, when suddenly closure follows a stimulation appropriately placed. This is apparently not due to cumulative stimulation, because sensitive areas can be found where response follows a first application almost invariably. Such regions are those which presumably involve the touching of the inner sets of sensory cells.

An attempt was made to obtain information on these points by causing a glochidium to close on a hair, and then observing where the tip of the imprisoned hair was placed. Great care must be exercised, as the hair tends to swing about the clasped region as a center, and then causes the tip to change its position. In the majority of cases, the inner halves of the valves being chiefly explored, the hair-tip after closure lay at the general location of the anterior pair of inner sensory cells. Possibly the structural relations are such that during stimulation the hair is guided to this spot with greater surety than to the posterior inner set.

It has already been noted that the half of the mantle away from the hinge is much less sensitive than the nearer half, and, furthermore, that the outer sensory cells are the only ones that can be satisfactorily seen in the living larva. It is a common experience to touch or rub the outer cells vigorously several times before eliciting a response. Due to the curvature and gaping attitude of the valves, and the consequent tendency of the glochidia to rotate, care must be exercised that in stimulating the outer cells a more proximal part of the hair does not touch the inner cells. However, it is apparently true that closure can be obtained under these conditions when only the outer hair cells are stimulated. It is perfectly certain that tactile sensitivity is not acute in this region, and the response tends to be slower and more



deliberate. It is of undoubted benefit to the organism that this condition should exist. A keener sensitivity near the hinge insures a more liberal 'bite' and a firmer hold. This facilitates encystment, which in fact may not proceed, in the hooked forms at least, if only a shred of host tissue is caught.

Whether closure ensues only in case a hair cell is touched is likewise difficult to determine. It is not easy to stimulate the most sensitive regions and be sure that no part of the hair has come in contact with the sensory cells, for the open glochidium tends to rotate when thus touched. After prolonged attempts to decide this point, I am led to conclude that one can stimulate between the hair cells and yet obtain contraction.

These results may be summarized in the statement that the hair cells are sensitive to tactile stimulation, the inner pairs far more so than the outer; yet it is believed that the glochidium may be responsive when the hair cells are not directly touched. The soft parts of the larva are delicate and the gentlest touch causes a deformation of the tissue, as is directly observable; for this reason it is not improbable that the application of a deforming pressure near a hair cell causes a traction which constitutes an effective stimulus. It is conceivable that closure normally follows a mechanical activation of the muscle, either directly or by remote traction on the mantle; nevertheless, the total experimental evidence does not favor such an interpretation.

In a few cases when touching glochidia by a succession of staccato prods (the hair being withdrawn before it could be caught), the valves closed but partially—perhaps three-quarters—and then slowly opened to the maximum. Possibly this is to be interpreted as an optimal stimulation applied at the limit of an area of indirect influence on the hair cells. Usually, however, the response is 'all or none.'

Spontaneous closure is sometimes seen among glochidia of a particular lot. This may involve jerky contractive movements which gradually bring the valves together, or it may be accomplished in a single movement by a quick or slow snap. In still other cases there are spasmodic partial contractions, the valves merely 'winking.' After closure, some sooner or later open again;

others shut almost completely and then open immediately. It is interesting to find that glochidia in such an apparently irritable condition do not have a noticeably augmented sensitivity to ordinary tactile stimulation.

It may be thought that since a hair, relatively small and hard, is not the sort of substance to which glochidia are destined to attach, other softer and larger objects might elicit still more ready responses. (Yet it is well to remark at this point that a thread or cord separated into its component strands to form a brush, and dragged through a suspension of glochidia for a short time, becomes clasped by many larvae (p. 488).) However, it is true that a softer, larger object that fits better between the valves produces superior results. Such a satisfactory agent is found in a tiny shaving of cork, cut by a razor to a sword shape, like a gill filament. Glochidia attach readily to the point or edges of this flexible object. The best responses are naturally obtained with a gill filament itself, the soft, lamellated tissue demonstrably conforming to the space between the valves. The increased responsiveness by this sort of treatment is probably dependent upon the stimulation of more sense organs, which, in its last analysis, is a question of surface area of contact. The theoretical possibility of a chemical influence from a gill will be considered later (p. 488); it need not concern us here.

*b. Lampsilis ligamentina* responds actively to stimulation with a hair, but probably not quite as sharply as *L. luteola*. These glochidia also attach to yarn unraveled into a brush and agitated with them. They likewise attach very readily to fine cork shavings.

*c. Lampsilis anodontoides* gave results very similar to *L. ligamentina*.

*d. Lampsilis gracilis* glochidia, although very small, respond rather promptly to stimulation with a hair. They are so small it is difficult to localize the stimulus accurately.

2. *Axe-head glochidia*. The species available was *Proptera* (*Lampsilis*) *laevissima* (figs. 5 and 6). The valves are extremely short in anteroposterior extent, and are capable of opening very wide; in this position they present an elongate oblong shape.

Exploration with a hair proves that they respond readily when appropriate regions are stimulated, the most sensitive spot being about one-third distant from the flaring ventral border. Stimulation near the hinge elicits responses of a slower, deliberate nature.

The narrowness of the valves makes the tactile stimulation with a hair less easy to apply than in many forms. To a thin shaving of cork, shaped like a gill filament, or to an actual gill filament, they respond more avidly. These objects fit better between the valves and come in contact with a broader surface than does the tip of a hair.

3. *Hooked glochidia*. The mature glochidia of *Symphynota complanata* and *Arcidens confragosus* are promptly reactive to a hair (figs. 1 and 2), particularly a blunt one, but like other forms they respond even more readily to a soft body offering greater surface. The movement of closure is, however, rather deliberate, and once shut the closure is permanent. In my experience neither are they as delicately sensitive, nor is the response as vigorous, as in some of the hookless forms. Why Lefevre and Curtis ('12, p. 155) (working largely with *Symphynota*) should draw the following conclusion, is not evident: "Hooked glochidia, in striking contrast with the behavior of the hookless forms, respond very actively to tactile stimuli. . . ."

*Anodonta corpulenta* is extremely sensitive to delicate tactile stimulation—possibly more so than any other form observed by me. To a hair it responds by a sudden, quick closure from its widely open resting position. It may then promptly relax, and, if the hair is withdrawn, give several snapping movements.

When these hooked glochidia become attached to a suitable object, such as a cork shaving, the closure is permanent.

It was attempted to determine whether the threshold to tactile stimulation is altered just before or after a spontaneous contraction, but no appreciable changes were detected.



*B. Photic excitation*

To determine whether conditions of illumination influence glochidial activity, ripe individuals of *Lampsilis luteola* were divided into two lots, one of which was subjected to darkness for seven hours, the other to bright, diffuse daylight for a similar period. Tactile tests revealed no difference in sensitivity between the two. Similar negative results have followed trials after illumination with strong and with weak light. Glochidia of several *Lampsilis* species were found not responsive to differences in light intensity through shading; this applies both to quiescent larvae and those in an irritable, 'snapping' condition.

There is a feeling among some experienced in mussel propagation that bright sunshine favors infections. Thus Howard ('14, p. 21), referring to the difficulties of artificial infection with *Quadrula ebeus*, writes: "The results were more favorable when the sun was out than during cool, cloudy weather and apparently better in sunlight than in shade." So far as they go, the experiments which I performed with the *Lampsilis* group do not support this view; yet the suspicion arises that if there really are better results with illumination, as described, they are referable to temperature rather than light. Incidentally, the conditions of infection in nature are not such that a correlative sensitivity to quantitative light differences would be generally useful. Dr. F. H. Reuling states that in his experience with the summer propagation of various lampsilids he has never been able to establish any correlation between ordinary diurnal variations of light and temperature, on the one hand, and the ease of obtaining infections, on the other.

*C. Thermal excitation*

Glochidia of *Lampsilia luteola*, cooled to 10° to 15°C., showed no perceptible decrease in tactile response. When the temperature was reduced to 3° to 5°C., the reactions were apparently duller and less vigorous. It is entirely probable that a time factor operates here; there was some evidence obtained to sub-



stantiate this. That protoplasmic activity should be correlated with temperature is in accord with general experience in animal conduct.

It is found in experimental propagative work that cold weather infections are not so successful as summer ones. Dr. A. D. Howard informs me that in winter he was unable to obtain an infection with *Lampsilis anodontoides* on the gar until the water was warmed. According to Dr. F. H. Reuling, summer infections are gained at 75°F. with obviously greater ease than, for example, at 65°F.; probably the explanation for this lies wholly in the variable respiration rate of fishes due to temperature. In extremely cold weather the decreased activity of the glochidia doubtless is a factor as well, yet some larvae which mature during the winter are notably active (p. 476).

#### *D. Other modes of excitation*

Glochidia, being non-motile, cannot express a response to such sources of stimulation as gravity, currents, and ordinary surface contact. Their common position on the bottom with gaping valves directed upward is of use to those that attach to fins and other external surfaces. The assumption of such an advantageous orientation is purely mechanical, due to the curvature of the open valves and the distribution of body mass.<sup>6</sup> It is observed sometimes that salt crystals introduced into a dish of glochidia cause those near the limits of the sphere of influence to partially contract, or 'wink,' several times before closing. This I have never observed with solution of salts. It seems probable that such a phenomenon is dependent on lines of diffusion extending from the salt crystals which for a time stimulate the glochidium unequally.

<sup>6</sup> It may be noted here that the valves of glochidia of *Anodonta corpulenta* are maximally opened to 180°; this results in the larvae's assuming positions with the valves directed either up or down.

*E. Chemical excitation*

The valves of a glochidium close in response to various chemical solutions. A common method of testing the ripeness of glochidia and their fitness for propagative infections is to add crystals of table salt to a sample of larvae and observe its effect. From observations on the action of blood and a few commoner salts, Lefevre and Curtis ('12) reported a closing response in both hooked and hookless forms. Since they believed the tactile sensitivity of hookless glochidia inadequate to cause attachment, it was assumed that the response in this group is at bottom chemical, induced by hemorrhages from abraded gill tissue. This view will receive attention on a later page (p. 486).

The method of procedure followed in these determinations was to place 50 to 100 active larvae on a slide in a water film just sufficient to allow their separation. They were then so flooded with the proper solution that the original water did not materially affect the final concentration. The limiting concentration adequate to produce responses and the promptness of the reaction at the lower dilutions furnished the criteria for comparisons.

1. *Hookless glochidia*. As in the other experimentation, the most intensive tests were made on the large glochidium of *Lampsilis luteola*. First, the range of chemical sensitivity was sought with a variety of reagents, and next, certain critical quantitative data.

*Acids**HCl:*

N/2 and N/25. All glochidia close at once.

N/100. All, or nearly all, close.

N/250. A few only respond.

*Acetic acid:*

M/25. All close but the response lags.

M/50. Same.

M/100. Relatively few affected.

The inferior stimulative power of acetic, as a type of organic acid, is well known.

*Picric acid:*

M/10. Most close at once; others only after a definite latent period. Closure may be quick, or slow and deliberate.

M/100. Practically no response for nearly two minutes, then closure begins progressively throughout the field, glochidia shutting slowly and evenly. A considerable number of initially closed larvae open after one or two minutes.

M/250. No closure observed in ten minutes, although glochidia still very responsive to touch. Opening of closed specimen observed as at M/100.

M/500. No effect in five minutes.

Picric acid was used chiefly as a representative of these substances bitter to human taste.

#### *Alkalies*

##### *KOH:*

N/100. All close immediately.

N/250. Response slow, many shutting late and with a deliberate contraction.

N/400. Response delayed for about thirty seconds, perhaps one-half closing during subsequent observation period.

N/600. Few only respond.

#### *Alcohols*

##### *Ethyl:*

8M. All respond immediately.

4M. All close, but in many the response is deliberate.

M/2. No effect.

##### *Methyl:*

20M. All close at once.

10M. A few respond promptly, but most very slowly and gradually.

3M. No effect.

It is interesting that the really effective solutions, 8M ethyl and 20M methyl alcohol, represent concentrations of 37 and 64 per cent, respectively. These are lethal doses.

#### *Sugars*

##### *Saccharose:*

4M. All close at once.

1M. Many become shut, but usually only after a distinct latent period. Most that close do so very gradually, some at an almost imperceptible rate.

M/4. No definite effect in ten minutes. Perhaps some had initiated contraction (compare 1M), but if so it had not progressed far.

##### *Dextrose:*

2M. No effect for several minutes, then contraction enters with such extreme slowness that it can be easily overlooked. Eventually all close.

1M. Same as in 2M, but even less rapid.

A 4M solution of saccharose represents a saturated syrup. Hence the action of the other various chemical substances employed is not significantly osmotic.

*Essential oils*

Oil of cloves and wintergreen, in saturated aqueous solution, cause glochidia to close with great promptness and vigor. These are odorous substances which possibly are effective as irritants.

*Salts*

A comparative test was made with the neutral halogen salts of potassium, to discover among other points the relative stimulative efficiency of the anions.

*KCl:*

N/100. All respond immediately.

N/250. Closure progressive during five to ten minutes.

N/400. Most close within ten minutes but some do so slowly and deliberately.

N/600. Some are closed at the end of ten minutes; most remain open.

*KBr:*

N/4. Effective at once.

N/50. All close, some a little tardily.

N/100. Affects samples from two mussels rather strongly; in another sample the response is weak, but quite general in five minutes.

N/250. Little or no effect.

*KI:*

N/25. All close.

N/100. Affects relatively few.

N/300. A very few close.

N/500. Practically no response; after an interval a few shut.

A comparison was also made of the stimulative efficiency of the kations of the alkali metals and also of the divalent kations, Mg and Ca.

*KCl:* (Vide supra.)

*NaCl:*

N/4. Effective.

N/10. All close, but rather slowly.

N/25. Closes half of one lot and all of another in ten minutes.

N/50. No response in ten minutes.

*LiCl:*

N/4. All closed, but after a distinct latent period of five to ten seconds or more.

N/10. One lot responded fairly promptly; another slowly, after several minutes.

N/25. After five minutes a few begin to contract, but the response is not general.

*MgCl<sub>2</sub>:*

5N. All close at once.

1N. Some shut immediately, the remainder gradually.

N/4. Same as 1N; action not rapid.

N/10. A few respond in one lot; practically none in another.



*CaCl<sub>2</sub>*:

5N. All close promptly.

1N. All close, but response begins only after one-quarter to one minute and is slow and deliberate.

N/4. No effect on one lot and practically none on another.

Arranging the results on page 481, the order of anion stimulating efficiency is found to be.



This coincides with the series found for Chiton (Arey and Crozier, '19; a discussion of certain other conflicting results appears in this paper as well).

The order of kation stimulative efficiency for the alkali chlorides is:



The difference between Na and Li is slight, but Na appears somewhat more stimulating. This, however, reverses the order of these kations established for a number of marine invertebrates, as well as their action on various protoplasmic processes (Arey and Crozier, '19). Including the divalent kations Mg and Ca, the series becomes (compare Crozier and Arey, '19):



An attempt was made to discover whether a chemical solution, too weak in itself to evoke a reaction from glochidia, will lower the tactile threshold by increasing the general protoplasmic irritability. HCl and NaCl furnished no positive evidence. It is possible that after treatment with weak KCl the tactile stimulus need not be so accurately localized directly in the sensitive areas as formerly; also with LiCl the response seemed perceptibly more ready. At best the increase is but quantitative and of no practical significance. Hooked glochidia, activated to repeated snapping by weak KCl (p. 483), were similarly tested, but it is not certain that their tactile threshold was thereby lowered.

Except at the highest concentrations, a distinct latent period characteristically occurs between the application of a chemical solution and the response; this is suggestive of a direct stimulation of muscle without nerve mediation.

2. *Axe-head glochidia*. *Proptera* (*Lampsilis*) *laevis* was tested merely with KCl in order to compare its relative sensitivity to this most active salt. There is little difference between the reaction threshold of *Proptera laevis* and *Lampsilis luteola*.

KCl:

N/100. All close promptly.

N/250. Some contract at once, the rest progressively during an interval of one to two minutes.

3. *Hooked glochidia*. For comparison with the hookless and axe-head types, the effect of KCl was tried on *Symphynota complanata*, *Arcidens confragosus*, and *Anodonta corpulenta*.

KCl:

N/100. All three glochidia were thrown into snapping contractions, some closing at once, others after several preliminary snaps. A certain number closed progressively by a series of jerks. *Anodonta* displayed by far the most vigorous snapping.

N/250. *Symphynota* and *Arcidens* closed rather promptly. The response in *Anodonta* was slower, perhaps due in part to retarded penetration through a certain amount of undissolved slime which encloses the larvae and is more resistant than that of the other forms.

N/500. *Symphynota* closed in about two minutes, *Anodonta* more tardily.

N/1000. *Symphynota*: After rapid pulsations all closed within two minutes; it is plainly more sensitive than the others. *Arcidens*: Some become shut in one to three minutes and nearly all within five minutes. *Anodonta*: Contractions are rapid and prolonged. At the end of five minutes many are closed, whereas others continue snapping.

N/2000. *Symphynota*: Some respond quite promptly; most of the remainder are shut in five minutes. [Distilled water, by itself, is without effect.]

It follows from these observations that the threshold of stimulation to KCl in hooked glochidia is far lower than in either the hookless or axe-head types. Doubtless diminished responses would have continued to considerably greater dilutions had trials been made. Yet N/2000 KCl is physiologically very dilute; it represents a 0.0037 per cent solution. The minimal concentration of KCl effective in the sensory activation of certain (marine) animals are (Arey and Crozier, '19): *Ascidia*, N/4; *Chromodoris*, N/10; *Synaptula*, N/40; *Chiton*, N/160; *Bal-*

anoglossus, N/200; Holothuria, N/500. For man the limiting concentration in taste lies below N/250, probably nearer N/100.

4. *Chemical excitation in nature.* In a previous section the conclusion was reached that all the glochidia possess a rather acute and nearly equal tactile sensibility. This is not in agreement with the statements of Lefevre and Curtis ('12). The data on chemical excitation, just presented, also show that, to KCl at least, the hooked forms are the more delicately reactive. At the outset, accordingly, the bare evidence would not appear favorable to the view which Lefevre and Curtis advanced, that the usual effective stimulus causing hookless glochidia to attach was chemical in nature, of hooked glochidia mechanical. Finding, moreover, that the blood of vertebrates causes the larvae to snap shut, they concluded that blood, through the activity of its salts, was the agent responsible for the attachment of hookless glochidia to gills. They write (p. 154):

Since the hookless glochidia, which are essentially gill parasites and, when taken into the mouth of the fish lodge among the gill filaments, produce abrasions of the delicate epithelium covering the latter, a more or less extensive hemorrhage from the blood capillaries occurs, as may be readily seen from a microscopic examination. It is therefore evident that blood exuding from the gill filaments in the immediate neighborhood of the glochidia must have the same effect as in our experiments, and, by exciting vigorous contractions of the adductor muscle, furnish an efficient stimulus in bringing about a firm and permanent attachment to the filaments. It is true that hookless glochidia will occasionally secure an attachment to the edge of fins and other external parts of the fish but it is quite evident that they are not adapted to such locations, as they rarely succeed in remaining there. It is possible that when they do become attached to the fins the closure of the valves is due to the presence of blood on the latter; but, since hookless glochidia occasionally close when touched repeatedly, the attachment in these situations is probably brought about by a sluggish response to contact with the edges of the fins. Their characteristic place of attachment, however, is the gill filaments, and this definite reaction to the fish's blood constitutes a most striking functional adaptation to the special habit of hookless glochidia as gill parasites.

Blood of vertebrates causes glochidia to close promptly. Adding the blood of fish, frog, or man to a watch-glass containing *Lampsilis luteola* glochidia, however, has never resulted in throwing them into "rapid and violent contractions, alternating with

relaxations," as Lefevre and Curtis describe for the forms they studied. On the contrary the blood diffuses very poorly. Those glochidia under its immediate influence usually contract with one vigorous snap; those near by remain unaffected. Unless mixed by hand, the sphere of influence of shed blood is restricted.

Fish blood is notorious for its rapid coagulation; in fact, it is even difficult to obtain successful smears because of this. When a clean cut is made in a gill filament, the end seals so quickly that the excised portion retains the blood within its vessels. Rapid tissue proliferation further covers the cut end. Such an excised filament, washed or not, introduced among glochidia, causes no closure unless brought in actual tactile contact with the soft parts of the larva. If the filament is now cut across with a needle, and the blood forced out, the glochidia enveloped in the blood mass usually close, but those even very close may not. After a time, if considerable blood has escaped, larvae some distance away may become affected, but this closure is usually tardy and lacks vigor.

The same authors state (p. 154) that "it was astonishing to see what a small quantity of the fish's blood was required to produce the reaction." Of course, the real effective factor is not the actual quantity, but the concentration. The following experiment on *Lampsilis luteola* will illustrate the efficiency of blood in causing the closing response:

Buffalo fish were bled and the blood defibrinated. After proving that the serum is effective alone, the blood was diluted with distilled water and the solutions thus obtained added to glochidia according to the method already described (p. 479.)

1 serum: 4 H<sub>2</sub>O. Glochidia close quickly.

1:8. All shut: some tardily.

1:16. All close, but most after a latent period.

1:32. A very few shut promptly; the remainder after a considerable latent period.

1:64. All respond after several minutes interval.

1:132. After about one minute some begin to shut; in five minutes perhaps half have closed.

This sensitivity is of about the grade shown by the hooked types to KCl alone, but is greater than *Lampsilis luteola* dis-



played to the same salt. Doubtless there is an additive ionic effect in blood serum.

Just how blood would be of use in causing glochidia to attach to gills is not clear. Lefevre and Curtis state that they "lodge among the gill filaments, produce abrasions of the delicate epithelium covering the latter, [and] a more or less extensive hemorrhage' from the blood capillaries occurs, as may be readily seen from a microscopic examination. It is therefore evident that blood exuding from the gill filaments in the immediate neighborhood of the glochidia . . . by exciting vigorous contractions of the adductor muscle" brings about attachment.

Is it true, in the first place, that glochidia, barely of macroscopic size, carried past the gills in the respiratory current, produce 'abrasions' sufficient to cause 'hemorrhages,' or 'exudations'? Zoölogists who have long studied experimental infection at the Fairport Station state they have never seen corroborative evidence for such a view. It certainly strains the credulity of less experienced observers. Admitting for the moment its reality, how would those light natural infections where few glochidia only may be encountered, be explained? It is, likewise, debatable whether exuding blood, assuming it were not too rapidly swept away by the relatively large-volumed respiratory current, would not close most glochidia before they were in a position to attach; there is demanded the combination of a fast-moving glochidium in the exact position to clasp gill tissue and the simultaneous activation of its adductor muscle. I do not believe that this view of excitation by blood, admitting it were based on fact, will commend itself to the reader. That subminimal concentrations of blood, by dilution in the respiratory current, would serve to sensitize glochidia appreciably to tactile contact, also finds no support in experimentation (p. 482).

Theoretically, there are more refined ways in which blood might act. It is found that glochidia clasp a fibrous bit of coagulum avidly. They also attach somewhat more readily to an alcohol-fixed gill filament, which has been washed two days and then smeared with blood or wiped in coagulum, than to a filament similarly treated except for the blood. It might be thought that

as glochidia bite into the gill tissue there would be a slight oozing about the rim of the valves, and that this fluid, spreading and coagulating on the adjacent epithelium, induces attachment chemically. Avoiding the question as to how the first larvae would gain attachment, the remainder of the supposition is not borne out by direct observation, not even when the valves compress, and sink into, gill tissue which includes a blood vessel.

It would appear that the tactile sensibility of glochidia is adequate to insure encystment. A chemical responsiveness is likewise highly developed, possibly because the organs of sensory reception are of a 'general' type (Arey, '18; but note the evidence for the direct stimulation of muscle, p. 482), yet the organism makes little use of it. To state dogmatically that a chemical response is not utilized during or after attachment, is, perhaps, hazardous. At least, on the basis of observation, it may be said that the facts can be explained satisfactorily on a tactile basis, and that if there is any chemical perception operative it is not through distance receptors (p. 488).

The results of quantitative studies on chemical activation may prove to be directly applicable in propagative work. At present the physiological fitness of glochidia is tested by adding crystals of common salt to a sample of larvae. The resulting solution is relatively of high concentration. It would be far more rational to test this sensitivity to an active salt, such as KCl, at appropriate limiting concentrations; thus for *Lampsilis luteola* (and perhaps for the mucket group, or even the *Lampsilis* class?) samples which close promptly in N/100 KCl (p. 481) would be sufficiently 'ripe' and 'active.' To what extent such procedure is practical or advisable, only experimentation can decide; theoretically, it promises much.

#### IV. THE MECHANISM OF ENCYSTMENT

That the tactile response is adequate to insure the attachment of hookless, as well as of hooked glochidia, is a statement amenable to proof. A hair moved about at random in a watch-glass containing glochidia will after a time have several larvae fastened to it. A thread or strand of twine separated into its component

fibers, brush fashion, will likewise pick up many glochidia in a minute.

Experiments were made as follows:

Two similar strands of twine were spread at the end into brushes. One was wet with water, the other with N/4 KCl or NaCl and the excess pressed out. Each was then dragged for one minute through dishes containing an equal number of glochidia, and washed gently for thirty seconds to remove larvae enmeshed but not actually attached. Many glochidia were fastened to each brush, but there was no constant or significant difference between the two.

In so far as this type of experiment is trustworthy, it shows no superiority for an object offering both tactile and chemical stimulation. Yet from the practical side, Howard ('14) states (p. 38) that when "fish were immersed in a solution of common salt (10 per cent by weight) before placing in the feeding tank, . . . it was shown that this treatment had the marked effect of causing rapid infection [of *Quadrula eburnus*] where previously it had been difficult to obtain." It will be recalled that somewhat improved responses were obtained when alcohol-fixed, excised gill filaments were first smeared with blood before offering to glochidia (p. 486). A preserved filament alone is, however, inferior tactilly to a living one; this difference is doubtless largely a matter of configuration and the physical state of the surface.

When a gill filament of a fish is excised, the cut surface instantly seals off the blood within the vessels and a prompt proliferation of tissue further serves to cover the end. In this way a filament is obtained clean of blood, even without washing. When the tip of such an excised filament is brought between the valves of a glochidium, the larva closes on it, provided actual contact has occurred. There is nothing to indicate a chemical activation or to suggest that "undoubtedly . . . this normal reaction is to chemical stimulation from the ions of protoplasmic salts diffused from the animal fluids of fishes' gills or bodies" (Howard, '14, p. 35).

Essentially identical results may be obtained with gill filaments first fixed in strong alcohol and thoroughly washed in water. The responses are nearly as ready as with the living gill; the reason for such inferiority as exists is obvious.



If an excised gill filament of a bass is agitated with glochidia in a watch-glass, many larvae become attached within a minute. The tissue bordering the edge of the valves begins to proliferate, and in a few hours the glochidium is overgrown or encysted. In one instance observed, cyst formation was so rapid that the large larva of *Lampsilis luteola* was enclosed in two hours. There is one marked difference, however, between ordinary encystment and that on an excised filament. In the latter, cellular proliferation does not stop when the glochidium is evenly enclosed by overgrown tissue; it continues until large, unsymmetrical cysts result.<sup>7</sup> Superfluous new growth also is the rule in the repair of simple cuts on excised filaments (fig. 15).

Cyst formation appears to be fundamentally a response of a reparative nature on the part of the gill tissues. To restore the epithelial continuity the glochidium is overgrown as a foreign body. That the reaction is not a response to some chemical or vital influence emanating from the glochidium has been proved by experiment:

Thin aluminum- or lead-foil was cut into oblong strips 0.15 mm. wide and 0.40 to 0.60 mm. long. When bent, these form a minute V- or U-shaped clip, smaller than many glochidia (p. 465). In fact, practice enables one to make clips smaller than can be handled successfully with ordinary instruments. Under binocular enlargement, such clips were attached by needles to excised gill filaments of the bass, *Micropterus salmoides*, firmly clamping the tissue. As figures 7 to 14 show, the clips become overgrown by stages counterfeiting glochidial encystment.

The stimulus operative in cyst formation, therefore, is believed to be essentially mechanical.<sup>8</sup> It appears, nevertheless, that there is some regulatory factor superimposed over the simple reparative tendency, and that when a filament is separated from

<sup>7</sup> It is interesting to record in this connection that the cysts on fishes made immune by repeated infection (Reuling, '19), are characteristically large and swollen.

<sup>8</sup> The reduction of the terms of encystment to the mere overgrowth of an abrading foreign body by host tissue may prove inadequate in some cases. It will be recalled that parasitic trematodes and copepods on the gills of fishes remain unencysted. Dr. A. D. Howard has observed the glochidia of *Quadrula heros* only partially encysted after several days' attachment to *Necturus*, which, however, is not the natural host for this species.



the fish, the normal inhibition, whatever it may be, that causes the healing process to stop at the appropriate point, is lost. In this sense, typical encystment depends on the integrity of the gill as a normal living entity attached to the body.

A reciprocal experiment was made to test whether the glochidium takes any part in stimulating cyst formation by a chemical or vital influence:

*Lampsilis luteola* glochidia were ground to a paste. A gill filament of the bass, *Micropterus salmoides*, was placed in a drop of this, another in a drop of tap-water for a control, and both set in a moist chamber. No positive results were gained that could be attributed to the glochidial mass. In some cases both the experimental gill and the control showed general surface proliferation to form investments, but the responses were inconstant and uneven; nevertheless, this phenomenon, by itself, deserves further attention.

Such experiments cannot be regarded as furnishing evidence that the glochidia induce cyst proliferation in any but a mechanical fashion. Moreover, Needham and Lloyd ('16) are not precise when they write (p. 291): "Whether it be the mussel that reacts to only a certain kind of fish substance, or the fish that reacts to form a cyst only for a certain glochidial stimulus is not known;" for not only do glochidia attach to any fish with appropriate configuration of parts, but also a cyst is usually formed in such instances; what does happen when a glochidium attaches to a non-host or immune host is the early loss of the cyst and contents by sloughing (Reuling, '19).

#### V. SUMMARY

The only response to excitation that glochidia can express is a closure of the valves by contraction of the adductor muscle.

Glochidia of all types respond promptly and vigorously to appropriate tactile stimulation. The half of the mantle nearer the hinge possesses the keenest sensitivity. This may be useful by insuring a more liberal bite of host tissue.

Tactile activation is probably mediated normally through the hair cells of the mantle, although closure can be accomplished without directly touching these.

Light or darkness is without influence on glochidial reactivity. There is no response to decreased illumination through shading.

Low temperature tends to dull the characteristic response.

Glochidia react to appropriate concentrations of acids, alkalies, alcohols, sugars, and salts, including substances which produce in man the various sensations of taste and smell. This activation is ionic, not osmotic. Subminimal chemical solutions do not notably increase irritability and thereby lower the threshold to touch.

The view that glochidia (particularly of the hookless type) regularly attach to the host through a chemical activation by blood, derived from gill hemorrhages, is untenable. There is likewise no evidence of any other effective chemical influence from the host. The tactile response alone is adequate to insure attachment.

The proliferation of gill tissue to cover an attached glochidium and produce a cyst may be studied on excised filaments. Fundamentally, it represents the reparative process of wound healing which restores the continuity of the epithelium. Cyst formation is not initiated or controlled by any vital influence of the glochidium, for it can be imitated by applying tiny metallic clips to a filament. Nevertheless, some normal regulatory factor fails to manifest itself in experiments upon excised filaments; overgrowth then produces large, malformed cysts comparable to the excess proliferation in these cases after simple incision.

The larval threads which entangle certain glochidia at spawning, the ropy mucus or gelatinous cords which embed others, and the mucus which causes the massing of still others, may each be useful in keeping the larvae together until they are established on the bottom and become free. In this way, the chances for a heavy natural infection of a host would be improved.

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## PLATES

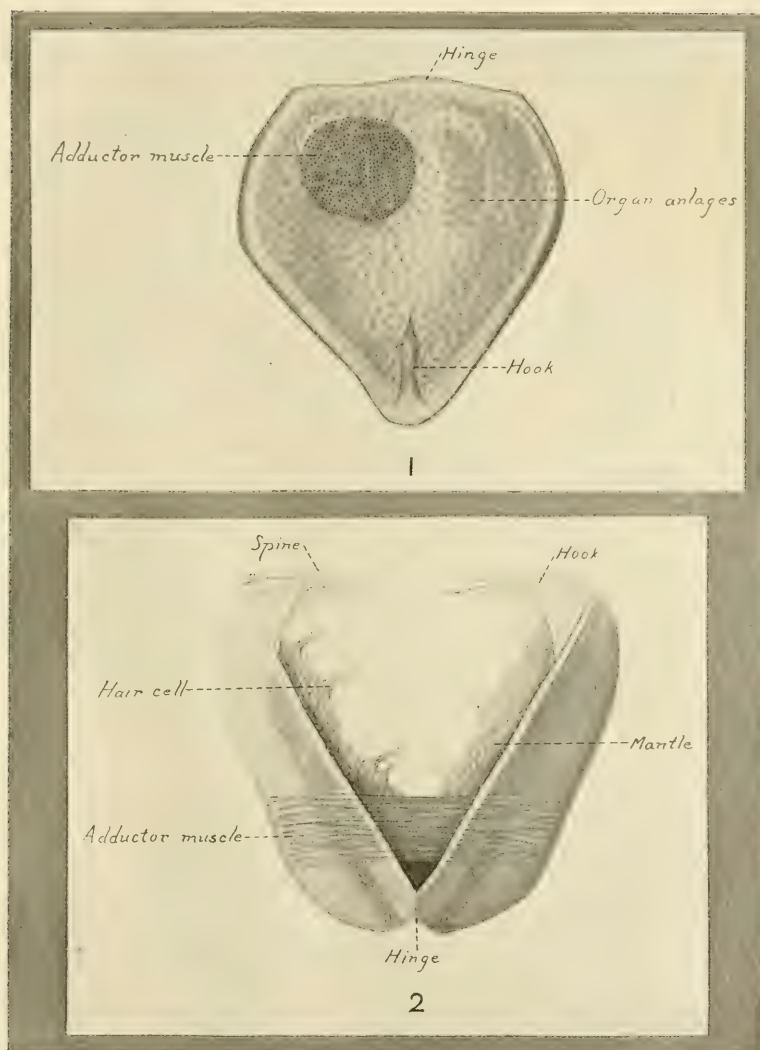


PLATE 1

EXPLANATION OF FIGURES

1 Hooked type of glochidium (*Symphynota complanata*). The valves are closed and the glochidium is viewed from the left side.  $\times 150$ .

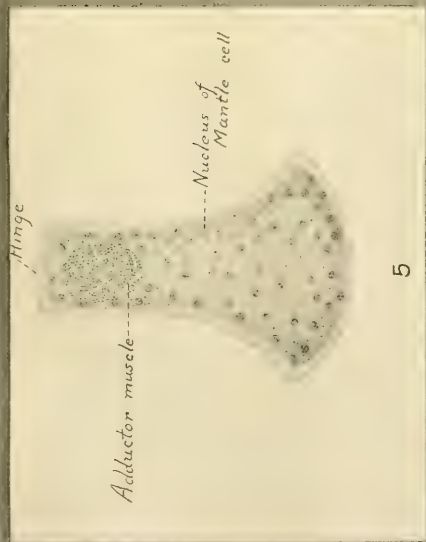
2 Hooked type of glochidium (*Symphynota complanata*). The valves are open.  $\times 175$ .



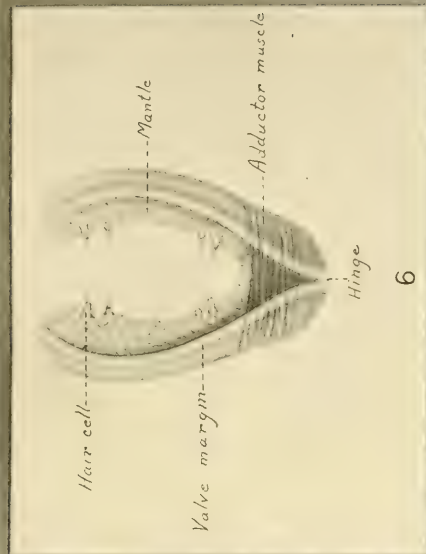
## PLATE 2

### EXPLANATION OF FIGURES

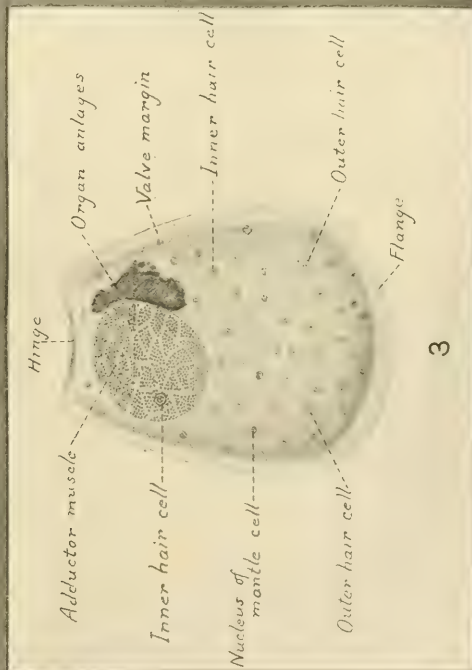
- 3 Hookless type of glochidium (*Lampsilis luteola*). The valves are closed and the glochidium is viewed from the left side.  $\times 150$ .
- 4 Hookless type of glochidium (*Lampsilis luteola*). The valves are open.  $\times 175$ .
- 5 Axe-head type of glochidium (*Proptera laevis*). The valves are closed and the glochidium is viewed from the left side.  $\times 275$ .
- 6 Axe-head type of glochidium (*Proptera laevis*). The valves are open.  $\times 250$ .



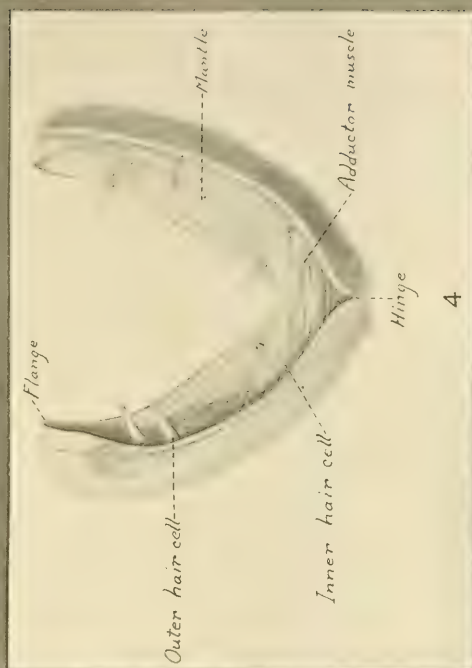
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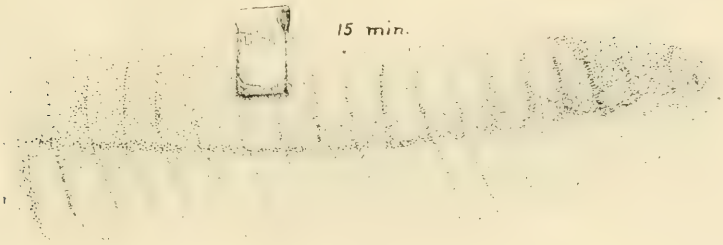


### PLATE 3

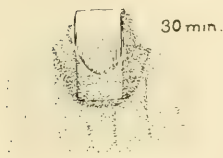
#### EXPLANATION OF FIGURES

7 to 14 Successive stages showing the overgrowth of an aluminum clamp, measuring 0.25 mm. by 0.12 mm., by the epithelium of an excised gill filament of the bass, *Micropterus salmoides*. The proliferation imitates cyst formation about living glochidia. At each stage is indicated the time elapsed from the beginning of the experiment.  $\times 50$ .

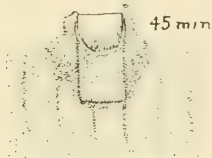
15 The repair of a wound made by simple incision in the excised gill filament of the bass. As with cyst formation on excised filaments there is excess proliferation.  $\times 50$ .



7



8



9



10



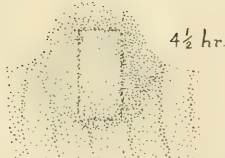
11



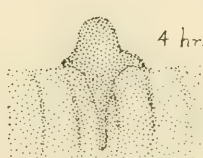
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